Immune activation at the lung epithelial barrier

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Summary

Dendritic cells (DCs) are commonly known as the most potent antigen presenting cells in several inflammatory diseases. In the lung the main function of DCs is to sample incoming antigens, transport them to the draining lymph nodes, where they will activate naive T cells to become effector T cells or regulatory T cells. The outcome of the response depends on the type of antigen, the type of instructing signals encountered in the periphery but also on the nature of the dendritic cell subsets presenting the antigen to T cells.

In **chapter 3** we have tried to understand the immune response developed towards antigens trapped in the lung vascular filter. We have shown that these antigens were presented to T cells by interstitial lung DCs in the mediastinal lymph nodes. In addition, we found that these interstitial lung DCs secrete monocyte-chemotactic protein-1 (MCP-1) after embolic antigen exposure, leading to the recruitment of monocytes. Deletion of interstitial DCs resulted in reduced inflammatory aggregates in the lung and antigen presentation in the MLN. Adoptive transfer of purified bone marrow monocytes into DC- depleted mice resulted in conversion of injected monocytes into monocyte-derived DCs and presentation of the antigen in the MLN.
In **chapter 4** we describe that bile acid ursodeoxycholic acid (UDCA) has immune regulatory properties by directly acting on the nuclear farnesoid X receptor on DCs. Treatment with UDCA during the secondary immune responses resulted in reduced features of allergic airway inflammation.
Recent studies revealed an important role for structural cells, especially epithelial cells, in allergic asthma. Lung epithelial cells express pattern recognition receptors (such as TLR4) and respond to allergens by producing DC-instructing factors. **Chapter 5** demonstrates that exposure of lung epithelial cells to HDM leads to release of uric acid (UA). Secreted UA acts as a danger signal to promote Th_2 immune responses to harmless inhaled antigens. UA-driven responses were mediated by DCs through Syk and PI3Kδ signalling pathway. Treatment of mice with uricase at the time of HDM sensitization reduced the features of allergic airway inflammation. **Chapter 6** shows that after HDM exposure, IL-1α is one of the key cytokines released by epithelial cells and acts in an autocrine loop to enhance Th_2 inflammatory responses. Signalling through the IL-1α/IL-1RI pathway induces the secretion of various chemokines and Th_2 instructing cytokines by lung epithelial cells. We found that IL-1α induced production of IL-33 and GM-CSF by lung epithelial cells. These two cytokines were found to be crucial in driving Th_2 immunity to HDM. Unexpectedly, we also found that epithelial-derived thymic-stromal lymphopoietin (TSLP) did not play a role in a mild model of HDM-induced asthma. However, the use of higher doses of HDM (inducing more severe asthma features) revealed a more important role for TSLP.

In conclusion, this thesis shows that lung DCs are important for the sampling of antigens in different lung compartments. In some cases, like upon exposure to inhaled allergen such as HDM, epithelial cell-derived factors control DC-induced responses. This crosstalk between epithelial cells and DCs involves cytokines such as IL-1α, IL-33 and GM-CSF but also endogenous danger signals like uric acid. Our findings place airway epithelial cells at the origin of Th_2 sensitization and therefore, airway epithelial cells can be considered as a therapeutic target for allergic asthma.

Samenvatting

Dendritische cellen (DCs) staan algemeen bekend als de beste antigeen-presenterende cellen in chronische ziektes zoals astma. De belangrijkste functie van DCs in de long is het fagocyteren van ingeademde antigen en deze te transporteren naar de drainerende lymfeklieren. Daar worden naieve Tcellen geactiveerd om vervolgens effector-T cel of regulatoire T cel te worden. De afweerreactie hangt af van het type antigeen, de instructies van het perifere weefsel en ook het type DC-subset dat het antigeen aan de Tcellen presenteerd.

In **hoofdstuk 3** onderzochten we de afweerreactie die gericht is op antigenen die zijn vastgelopen in de filterende haarvaten van de long. We laten zien dat deze antigenen aan T cellen werden gepresenteerd in de mediastinale lymfeklieren (MLN) door interstitiële long-DCs. Ook vonden we dat deze interstitiële long DCs monocyte-chemotactisch protein 1 (MCP-1) uitscheiden na de blootstelling aan embolische antigen, hetgeen leidde tot de aantrekking van monocyten uit het bloed. Het verwijderen van de interstitiële DCs liet een reductie in de ontstekingshaarden zien alsmede minder antigen presentatie in de MLN.

In **hoofdstuk 4** laten we zien dat het galzuur ursodeoxycholic acid (UDCA) de eigenschap heeft om immunologische reacties te beïnvloeden door te binden aan de farnesoid X receptor op DCs. Behandeling met UDCA tijdens de secundaire afweerreactie leidde tot een vermindering van de allergische luchtwegontsteking.

Recentelijk is aangetoond dat er een belangrijke rol bestaat voor structurele cellen, met name epitheelcellen, in allergisch astma. Epitheelcellen van de longen brengen patroon herkenings receptoren (zoals TLR4) tot expressie en reageren op allergenen door DC-instruerende factoren vrij te maken. **Hoofdstuk 5** laat zien dat blootstelling van long epitheelcellen aan huisstofmijt (HDM) tot de secretie van urinezuur (UA) leidt. Het vrijgekomen UA reageert als alarmsignaal om Th_2 reacties op ingeademde antigenen te versterken. Voor UA-geïnduceerde reacties zijn DCs van belang via Syk en PI3Kδ. Behandeling van muizen met uricase bij het ontstaan van astma, verminderde de eigenschappen van allergische luchtwegontsteking. **Hoofdstuk 6** laat zien dat na de blootselling aan HDM, IL-1α een van de belangrijkste cytokines is, dat wordt vrijgesteld door epitheelcellen en op een autocriene manier Th_2 ontstekingsreacties versterkt. Binding van IL-1α aan IL-1RI leidt tot de secretie van verschillende chemokines en Th_2-instruerende cytokines door long epitheelcellen. In deze experimenten vonden we dat IL-1α de productie van IL-33 en GM-CSF door epitheelcellen stimuleerde. Deze cytokines zijn cruciaal gebleken in het ontstaan van Th_2 reacties op HDM. We vonden onverwacht ook dat epitheelcel-afkomstig thymic-stromal lymphopoietin (TSLP) geen rol speelde in een mild astma-model. Maar als hogere dosissen van HDM werden gebruikt (Hetgeen leidt tot een ernstigere vorm van astma) was er een belangrijke rol voor TSLP weggelegd.

Tot besluit, dit proefschrift laat zien dat long-DCs belangrijk zijn voor het opnemen van allergen in verschillende compartimenten. Voor de interactie tussen epitheelcellen en DCs zijn cytokines zoals IL-1α, IL-33 en GM-CSF van belang, net als endogene alarmsignalen zoals UA. Onze bevindingen plaatsen het luchtwegepitheel aan het begin van de Th_2 sensitizatie en daardoor zijn long epitheelcellen een potentieel doel voor toekomstige therapieën in allergisch astma.

Introduction

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Introduction

Allergic asthma

It has been estimated that 300 million people are affected by asthma worldwide. The incidence of allergic sensitization and asthma has increased dramatically in developed countries over the last 50 years. Even more alarming is the ever increasing severity of the disease causing about 250.000 deaths per year [1]. Asthma is a chronic immune-mediated inflammatory disorder of the airways, characterised by attacks of coughing, wheezing and breathlessness due to eosinophilic airway inflammation, airway wall thickening, mucus secretion by goblet cell metaplasia, bronchoconstriction and airway hyperresponsiveness to non-specific stimuli. In allergic asthmatics, airway inflammation is initiated by specific triggers (inhalation of allergen) and exacerbated by non-specific triggers (like air pollutants and viral infection). These patients have elevated levels of allergen-specific IgE in serum and a positive skin prick test to common allergens. The presence of allergen-specific IgE is a strong risk factor for developing allergic asthma, on the background of genetic predisposition. GWAS studies in asthma have found multiple SNPs in genes that deal with host-immune system interaction (like NOD1, TLR-2,-6 and-10, sCD14), antigen presentation (HLA-DR, DENND1B), immune regulation (IL4R, IL13, IL12B, GATA3, STAT6, FCERIB), epithelial cytokine (receptors) (GM-CSF, IL-33R, IL1R, TSLP), epithelial barrier function (Fillagrin, GPRA, DPP10, Protocadherin 1, Defensin beta 1), tissue repair response (ADAM33, COL29A1, ALOX5, NOS1), bronchoconstriction (ADRB2, PDE4A, GPRA), and unfolded protein response (ORMDL3).

In the mid-1980s, Mosmann *et al.* published a landmark paper that characterized T-helper $(Th)_1$ and Th_2 subsets [2]. Over the past 30-years this Th_1-Th_2 paradigm has dominated the asthma research. As such, it has become clear that the immune response to inhaled allergens is characterized by an aberrant Th_2 lymphocyte response that has the potential to cause the features of asthma, through the production of Th_2-type cytokines like IL-4, IL-5, and IL-13 [3]. Subsequently, a large body of data from animal models in which CD4 T cells and these Th_2-type cytokines have been individually neutralized suggested that Th_2 cells can promote allergic airway inflammation. This view has been extended by the discovery of other T cell subsets, such as Th_{17}, Th_9, and regulatory T cells (Treg). However, the fact that Th_2-like eosinophil-rich responses could also be induced in mice lacking T and B cells, suggests a potential role for other cells (so called innate lymphoid cells or ILCs) during allergic immune responses [4]. Innate lymphocytes type 2 (ILC-2) are also known as nuocytes or natural helper cells and produce copious amounts of IL-5, IL-9 and IL-13. In mice, ILC2s derive from committed $T1/ST2^+$ pre-ILC2s that develop from common lymphoid progenitors in the bone marrow under the influence of IL-33 and/or IL-25 but not TSLP. As most experiments looking at the role of ILC2s have been performed in mice lacking T and B cells, it is still unclear what the precise role of these cells in asthma is. Therefore, most investigators still agree that allergic type inflammation is mostly controlled by adaptive CD4 T lymphocytes that secrete IL-4, IL-5 and IL-13, and require antigen presenting cells for their activation.

Antigen presentation by dendritic cells

The lung is continuously exposed to foreign matter (allergens, microbes, diesel particles etc.) by inhalation or sometimes via the bloodstream. Allergens and pathogens need to bridge physical barriers formed by lung stromal cells to be recognized by the host immune system. Most inhaled particles are immunologically inert, and therefore the usual outcome of their inhalation is tolerance. In the absence of inflammatory triggers, dendritic cells (DCs) that take up these harmless antigens do not properly express costimulatory molecules and consequently fail to reach the threshold necessary to induce T cell activation and instead induce an abortive T cell response.

DCs are the main antigen presenting cells (APC) of the immune system [5] situated near endothelial cells and lung epithelial cells under homeostatic conditions [6]. They are essential for priming and Th_2 differentiation of naïve T cells towards aeroallergens [7]. DCs play a central role in the initiation of primary immune responses and enhancement of secondary immune responses [8, 9]. Inhaled allergens are captured by DCs through endocytosis. The captured allergens are processed and generally presented by major histocompatibility complex (MHC) class II molecules to $CD4^+$ Th cells [5]. DCs that have captured antigen subsequently migrate to the T cell area of mediastinal lymph nodes (MLN) where they report the inhaled antigen to recirculating T cells (**Figure 1**). Under certain conditions, lung DCs can also capture antigens from infected cells and crosspresent this to CD8 T cells, thus mounting antiviral immune responses [10].

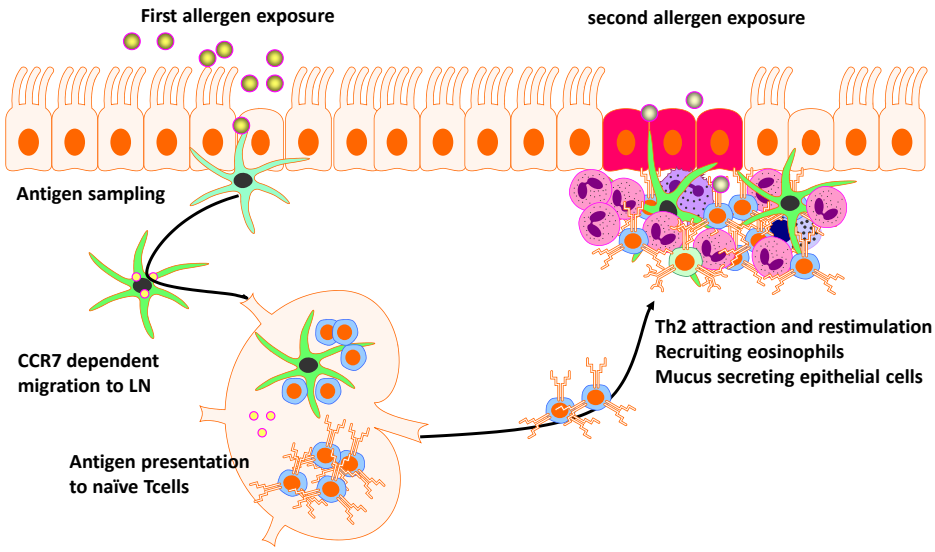

Figure 1: Dendritic cells situated just underneath the airway epithelial cell layer sample for the presence of inhaled allergens. Upon recognition and activation they migrate to the draining lymph node, while they process the antigen. In the mediastinal lymph node (MLN) DCs activate the naïve T cells. Subsequently, primed effector T cells leave the MLN, but can be recruited to the site of a second allergen exposure, where DCs release chemokines and cytokines to recruit and activate the extravasating effector T lymphocytes to mount the immune response to allergens and cause allergic type inflammation.

While processing recognizing allergens, DCs acquire a mature phenotype, meaning that they upregulate CD80, CD86 and CD40 costimulatory molecules and acquire the capacity to stimulate an effector response in dividing T cells [11-14]. DCs become a reporter of their earlier microenvironment and have the potential to induce a polarized Th_1, Th_2 or Th_{17} type of response [15, 16]. Many factors are decisive in the process of T-helper polarization. The type of antigen, presence of a microbial matter, route of exposure and genetic background of the host, all play an important role [17, 18]. These signals are translated, at the level of the APC, into signals that can be read by T cells. The most critical defining factors in this translational step are the type of DCs (conventional $CD11b^+$ cDCs or $CD103^+$ cDCs, plasmacytoid DCs and monocyte-derived DCs [19], see below) involved in the interaction with T cells, the type of co-stimulatory molecules expressed on the surface of the DCs, and the secretion of polarizing cytokines by DCs or presence of polarizing cytokines in the microenvironment during antigen presentation [20, 21]. DCs not only activate the immune cells, but also tolerize lymphocytes to prevent autoimmune responses. Tolerance is a feature of lung DCs in steady state, and is the best shown in mouse models of allergic asthma where ovalbumin (OVA) is used as an allergen. If OVA is applied without an adjuvant, such as aluminium hydroxide at the first exposure, no sensitization will occur [22], and an active counterregulatory response made up of Treg cells can be induced [21, 23]

Dendritic cell subsets

The main function of DCs is to initiate antigen-specific adaptive immune responses to foreign antigens that reach the tissues and to maintain tolerance to self-antigens. This is in contrast to macrophages whose main role is to detect and engulf damaged and dying cells, phagocytose opsonized microbes and promote tissue repair [24]. The role of DCs in adaptive immunity relies on their ability to process and present foreign antigens in complexes of peptide and MHC molecules on the cell surface, and their ability to migrate to the tissue-draining lymph nodes to activate T and B cells [25-27]. The emerging field of studying DC subsets in the immune response is very active. As a result, nowadays several DC subsets have been described, which vary per tissue and expression of several surface markers [28, 29]. Mouse lung DCs express the integrin CD11c as well as intermediate or high levels of MHC molecules on their surface. These markers are also expressed by alveolar macrophages but they can be distinguished based on autofluorescence [30]. Differentiation of DCs and macrophages in mice starts in the bone marrow. Hematopoietic stem cells (HSCs) give rise to granulocyte-myeloid (GMP) and lymphoid (LP) committed precursors (**Figure 2**). GMPs are precursors of monocytes, some populations of macrophages, and common DC precursors (CDPs). Subsequently, CDPs give rise to preclassical dendritic cells (pre-cDCs) and pDCs which are uniquely able to produce large amounts of the antiviral cytokine interferon-α and initiate T cell immunity to viral antigens [6, 31]. Pre-cDCs circulate in blood and enter lymphoid tissue, where they give rise to $CD8\alpha^+$ and $CD8\alpha^-$ cDCs [32]. These Lymphoid tissue–resident cDC subsets are functionally specialized, $CD8^+$ cDCs are best in cross-presentation of cell-associated antigens to $CD8^+$ T cells, whereas $CD8^-$ cDCs are the most potent at stimulating $CD4^+$ T cells [33]. In nonlymphoid tissues pre-cDC give rise to $CD11b^+$ and $CD103^+$ cDCs. Similar

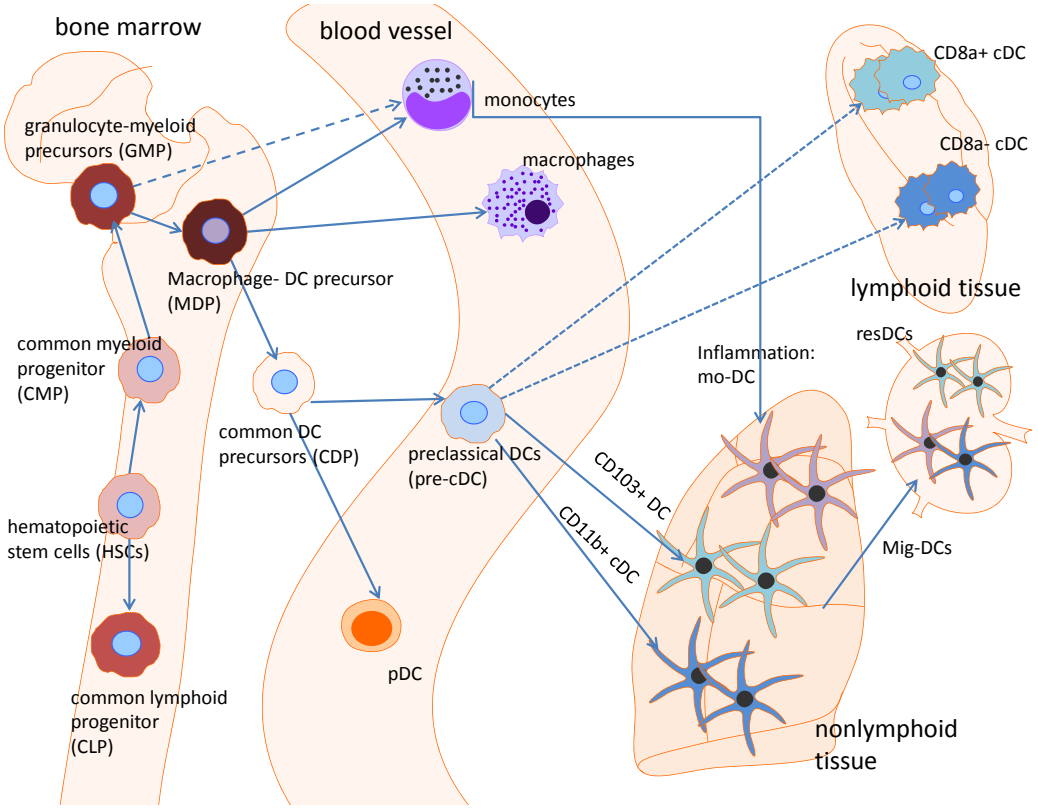

Figure 2: Hematopoietic stem cells (HSCs) give rise to common myeloid (CMP) and common lymphoid (CLP) committed precursors. CMP can differentiate into granulocyte-myeloid precursors (GMPs) which could be precursors of monocytes,and macrophage-DC precursors (MDP). MDP give rise to monocytes, some populations of macrophages, and common DC precursors (CDPs). CDPs give rise to preclassical dendritic cells (pre-cDCs) and plasmacytoid DCs (pDCs). Pre-cDCs circulate in blood and enter lymphoid tissue, where they give rise to $CD8\alpha^+$ and $CD8\alpha^-$ cDCs. In nonlymphoid tissues pre-cDC give rise to $CD11b^+$ and $CD103^+$ cDCs. Under inflammatory conditions monocytes can differentiate into monocyte-derived DCs (mo-DCs). DCs in the nonlymphoid tissue are able to migrate to the lymph nodes (Mig-DCs) where they join resident DCs (resDCs).

to lymphoid-tissue $CD8^+$ cDCs, nonlymphoid-tissue $CD103^+$ cDCs are efficient cross-presenters of cell-associated antigens and are the most potent at stimulating $CD8^+$ T cells. Our group showed that under lung-inflammatory conditions $CD11b^+$ cDCs are prone to sensitize to clinical relevant allergens such as house dust mite (HDM). Using markers CD64 and MAR-1, monocyte-derived DCs (mo-DCs) and $CD11b^+$ cDCs could be separated. HDM-specific Th_2 immunity is induced by $CD11b^+$ cDCs, while mo-DCs mainly produce chemokines and allergen presentation during the challenge phase [19]. Studies from our group have demonstrated that tolerance is induced by particular DC subsets; pDCs suppress T cell effector generation. Strikingly, in the absence of pDCs, exposure to harmless antigen led to Th_2 cell sensitization and features of asthma [21]. If pDCs promote tolerance and cDCs promote immunity, it is logical to assume that the balance between both subsets is tightly controlled. In support, administration of Flt-3 ligand, a cytokine that induces the differentiation of pDCs, in sensitized mice reduced all features of asthma [34]. Interestingly, we have shown that pDCs are anti-inflammatory irrespectively of their maturation state, however their protective effects are mediated through programmed death (PD)-1/PD ligand 1 interactions [34]. Current research focusses on progenitors and specific expression of transcription factors in between DC subsets [35, 36]. This information could be useful to specifically target a subset of DCs in inflammatory diseases.

Allergen recognition in the airways involves pattern recognition receptors

Over the last years, more focus has been put on dissecting the molecular mechanisms underlying allergenicity and Th_2 sensitization. Allergens are a complex mixture of biologically active molecules such as proteases, chitins [37], glycans, endotoxin, and a variety of still unidentified components. It has become increasingly clear that most of the environmental allergens express pathogen associated molecular patterns (PAMPs) that might trigger innate receptors such as Toll Like receptors (TLRs), NOD like receptors and C-type lectin receptors [38]. Signalling through TLRs strongly activates DCs to upregulate co-stimulatory molecules (CD80 and CD86) and to produce pro-inflammatory cytokines (TNFα, IL-1, IL-6, and IL-12) [39, 40]. PRRs 'sense' bacterial products and activate intracellular cascades that lead to inflammatory responses [41, 42]. PAMPs sensed by host inflammatory cells early during infection, are potent stimuli for innate immunity and are often referred to as 'exogenous danger signals' (**table 1**). Contamination of allergens with lipopolysaccharide (LPS) [39] or in combination of environmental exposures (respiratory viruses, air pollution or cigarette smoke) might pull the trigger in DC activation [43, 44].

	Dendritic cells	Lung epithelial cells
PAMP receptors		
Toll like receptors	TLR1-10	TLR1-6
Intracellular receptors	NLRs, TLR 3,7,9	NLRs, TLR 9
C-type lectin receptors	Dectin-1, -2, DEC205, BDCA-2 Macrophage mannose receptor	
RIG-I-like receptors	MDA5, LGP2	MDA5
Protease activated receptors	PAR 1-3	PAR 1-4
DAMP receptors		
Complement receptors	hCR1, hCR2, hCR3, mC3aR, mC5aR	mC3aR, mC5aR
Prostanoid receptors	DP1, EP2, EP4, IP	
Neuropeptide receptors	NK1, CGRPR	
Purinergic receptors	P2X, P2Y	P2X, P2Y
HMGB1 receptor	RAGE	RAGE
Heat shock protein receptors	CD14, CD36, CD91	

Table 1: examples of pathogen associated molecular pattern (PAMP) and damage associated molecular pattern (DAMP) receptors expressed on dendritic cells and lung epithelial cells.

Interestingly, polymorphisms in several pattern recognition receptors (PRRs) have been found associated with allergen sensitization and asthma [45]. As an example, both chitin that is present in HDM and cockroach frass are able to induce T cell responses that are TLR2- myeloid differentiation primary-response protein 88 (MyD88)-dependent [46-48]. Although both compounds trigger the same TLR, the outcome of induced immune response differs dramatically. Indeed, chitin was found to induce Th_2 responses and to promote allergen sensitization, whereas cockroach frass appeared to be protective against the development of allergic airway inflammation [46, 49]. We have shown that Der p 1, the major allergen from HDM, has proteolytic enzymes that can directly activate DCs and epithelial cells, promoting Th_2

sensitization [20]. It has been demonstrated that water-soluble mediators of birch-pollen promote their Th_2 potential and change the chemokine receptor profile of human DCs, thus favouring the attraction of Th_2 cells to the site of allergic inflammation. The water-soluble extract from birch pollen consists predominantly of E_1- phytoprostanes (lipids), which resemble the structure and function of prostaglandin E_2. These findings support the hypothesis that pollen grains themselves appear to contain important signals to pave the way towards a Th_2-dominated immune response [50, 51].

A possible explanation in the recognition of complex allergens involves triggering of several PRRs and signalling pathways, since some allergens can also trigger TLR4. Kim Bottomly's group has shown that the low amounts of LPS in the model allergen OVA lead to TLR4/MyD88-dependent Th_2 responses in the airways [52-54]. The fact that the TLR4/MyD88-axis was able to mediate a Th_2 responses was somewhat surprising since LPS, the prototypical ligand for TLR4, had been shown to protect from Th_2 sensitization [52]. However, the involvement of TLR4 signalling in mediating Th_2 responses has been recently supported by others using relevant allergens, like HDM, to induce Th_2 sensitization [55, 56]. TLR4 triggering by allergens has often been attributed to their endotoxin content [52], although there seems to be a more intimate link between allergic sensitization and TLR4 signalling. Surprisingly, the endotoxin contamination of HDM extracts used to induce allergic asthma are in the subnanogram range, much lower than the dose described promoting Th_2 responses to OVA [55]. Suggesting another molecule contributing in TLR4 signalling by HDM on epithelial cells. It has been reported that Derp 2, one of the major allergens of HDM, is a functional homolog of MD2, the LPS-binding member of the TLR4 signalling complex [57]. As such, Derp 2 might promote or facilitate TLR4 signalling. Other studies have shown that HDM can trigger TLR4 and Dectin-2 directly on DCs. Indeed, allergens such as house dust extracts or cockroach frass induced DC maturation and promoted the production of LTC4 [58], pro-inflammatory cytokines IL-6 and IL-23, and lowered levels of IL-12p70. The activation of DCs by HDM relied on downstream MyD88 signalling, although others have shown the involvement of IRF3 and Syk signalling [58, 59]. It is important to note that TLRs are not the only PRRs triggered by environmental allergens. In fact, some airborne allergens contain proteases or glucans that can facilitate allergen sensitization by stimulating various PRRs (protease activated receptors, C-type lectins) on various immune cells [60-63].

DAMPs stimulate DC-induced sensitization

Oxidative stress or tissue damage can trigger inflammation even in the absence of pathogens. Inflammation triggered by tissue damage in the absence of infection is often referred to as sterile inflammatory response. It is now appreciated that immune cells react to molecules released by injured or necrotic, but not apoptotic cells [64]. These molecules alert immune system of an impending danger, and are therefore also referred to as ´alarmins´, 'endogenous danger signals' or danger associated molecular patterns (DAMPs) [65]. These DAMPs interact with PRRs (shared with PAMPs) contribute to the induction of inflammation by recruitment of innate inflammatory cells. The actual repertoire of DAMPs in damaged tissues can vary greatly depending on the type of cell (epithelial or mesenchymal) and injured tissue. A few danger signals are heat shock proteins (HSP), high mobility group box 1 (HMGB1) protein, uric acid (UA) and adenosine triphosphate (ATP). DAMPs can be intracellular proteins secreted actively

through nonclassical pathways and endowed with inflammatory activity so-called leaderless secretory proteins (LSPs). These LSPs can be released by dying cells and behave as DAMPs. HMGB1 is a prototypical LSP that is passively released by injured or necrotic cells, or by immune cells in response to endotoxin promoting tissue inflammation [66]. A study on PBMCs however showed that HMGB1 alone cannot induce detectable levels of IL-6, except after co-administration of LPS, CPG-ODN, PAM3CSK4 or IL-1 [67]. Compelling evidence suggests that a tight collaboration between PAMPs and DAMPs is needed to start an immune response to allergens [68, 69]. In contrast to necrotic cells, apoptotic cells retain HMGB1 in their nuclei and therefore do not initiate inflammation [70]. These data suggest some similarities between infectious and sterile inflammation, since PAMPs and DAMPs seem to share PRRs [42].

Alum-induced Th_2 responses

One recent illustration of the potential implication of endogenous danger signals to the process of allergic sensitization comes from our studies on the mechanism of action of alum adjuvant. Alum is used in mouse models of asthma as a prototypical Th_2 adjuvant, whose mechanism of action is poorly understood. Alum added to DCs *in vitro*, poorly activates APC function with the notable exception of IL-1β induction [71]. *In vivo* however, alum strongly recruits and stimulates inflammatory DCs and boosts their potential to induce Th_2 responses, associated with production of bio-active IL-1β. We found that alum induces the release of UA, an endogenous danger signal released by dying cells or cells exposed to oxidative stress [72]. UA is known to induce the release of IL-1β, to promote Th_2 polarizing responses by DCs and induces IgG_1 [73]. The release of IL-1β requires the presence of a TLR agonist, IL-1 receptor I or TNF receptor I/II signalling acting on APCs to promote activation of NF-κB, subsequently leading to transcription and translation of pro-IL-1β [74]. Pro-IL-1β is cleaved by caspase-1 in the cytoplasm, whose activation in turn depends on triggering of a NLR, NALP3 (also known as cryopyrin), via endogenous danger signals [75, 76] leading to the release of bio-active IL-1β (**figure 3**). Recently, NALP3 activation was shown to in cells undergoing necrosis *in vitro* and *in vivo*, resulting in the production of mature IL-1β [77]. In addition, extracellular ATP has been known for years to activate caspase-1, and several studies have demonstrated the requirement of $P2X_7$ receptors (in a complex with pannexin-1) for ATP-induced caspase-1 activation and subsequent IL-1β maturation [78, 79]. Besides, *in vivo*, UA-mediated Th_2 cell development can act as an additional trigger. Certainly UA promotes the development of Th_2 responses when added to DCs *in vitro* [73, 80]. Therefore it is no surprise that mice deficient in NALP3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and caspase-1 have a defect in crystal-induced IL-1β secretion and fail to mount alum-induced Th_2 mediated inflammation *in vivo* [80, 81]. However this finding has been debated and it seems that alum induces the formation of the NALP3 inflammasome and requires the ASC protein leading to activation of IL-1β [82, 83]. But NALP3 activation was not necessary for the alum-induced adjuvanticity [81,161,162]. Cleaving of pro-IL-1β into its bio-active form does not occur only intracellularly by NLR activation. Double stranded DNA which is released by necrotic cells, is also potently able to induce caspase-1 activation as soon as it is cytosolic. Recently, PYHIN (pyrin and HIN domain-containing protein) family member absent in melanoma 2 (AIM2) is described as a receptor for cytosolic DNA to regulate caspase-1 induced

IL-1β maturation [84], via noncanonical activation of NALP3. AIM2 is the first non-NLR family member forming an inflammasome and is composed of AIM2, ASC and caspase-1 [164,165]. Neutrophils which are attracted to inflammatory sites, secrete proteinase-3, an enzyme which is able to cleave pro-IL-1β extracellularly. Other proteases such as elastase, matrix metalloproteases, granzyme A and the mast cell chymase also generate active IL-1β [85]. Therefore not all IL-1β-induced responses are necessarily NALP3 dependent. Another complicating factor is that many experiments addressing the role of caspase-1 in caspase-1 deficient mice are flawed by the presence of an additional mutation in caspase-11 in these animals. Therefore, many experiments addressing the *in vivo* importance of caspase-1 will have to be performed again in mice with an exclusive caspase-1 deficiency [86]. Moreover, caspase-11 is able to activate caspase-1 in response to non-canonical stimuli such as *E.coli* and cholera toxin B [86]. It is also possible that the dependence on the Nlrp3/ASC/Casp1 axis depends on the site of primary allergen exposure. Indeed, skin sensitization to HDM seems to require this pathway, whereas sensitization via the lungs does not [87].

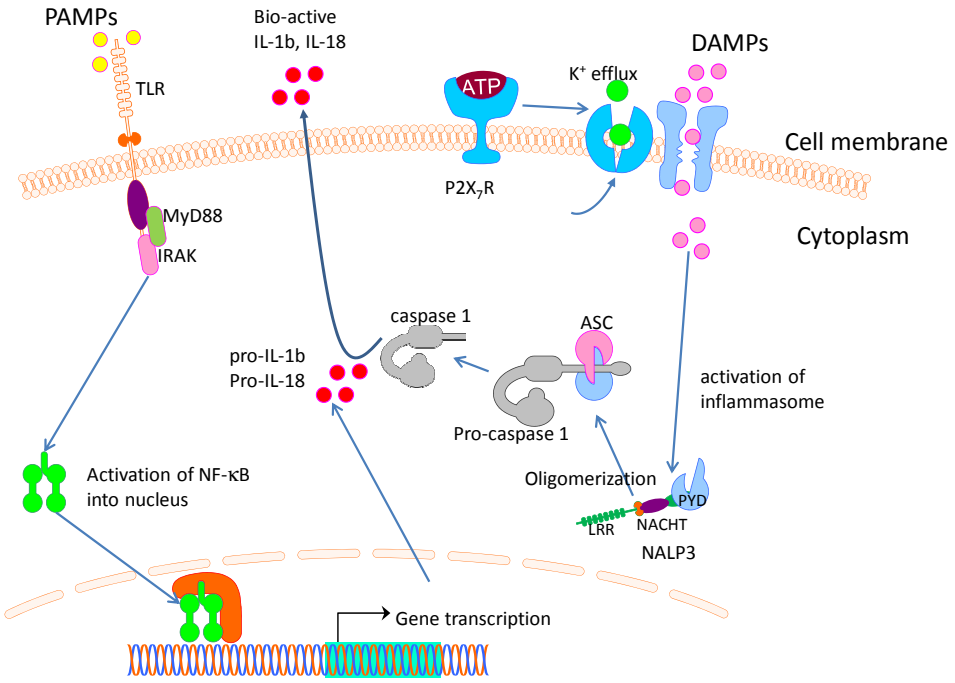

Figure 3. Schematic representation of classical NALP3 inflammasome activation by lipopolysaccharide (LPS) and adenosine triphosphate (ATP) followed by release of inflammatory cytokines. Microbial molecules, such as LPS, bind to TLR4, which recruits MyD88. MyD88 will in most cases bind to IL-1 receptor associated kinase (IRAK), which will lead to nuclear factor-kB (NF-κB) activation. NF-κB is a transcription factor which is responsible for the transcription of inflammatory cytokines. Pro-IL-1β and pro-IL-18 are then released in the cytosol. The second signal activating the $P2X_7$ receptor by ATP, leads to large pore formation by pannexin-1. DAMPs (for example uric acid) are than able to enter the cytosol and activate the NALP3 inflammasome [78–80]. Activated NALP3 consists of a pyrin domain, a NACHT domain and 11 leucine-rich repeat (LRR) domains. Activated NALP3 oligomerizes, recruits ASC and pro-caspase-1, which results in caspase-1 processing and activation. Active caspase-1 subsequently cleaves the precursor forms of IL-1β and IL-18 leads to release of the bio-active cytokines

Crosstalk between epithelial cells and lung DCs

Although DCs can directly respond to allergens in vitro, one major caveat is that most of the studies performed have used bone marrow-derived DCs (BMDCs) in mice or monocyte-derived DCs in humans to mimic lung DC responses. The direct effect of allergens *in vivo* on tissue- DCs remains unclear. There is evidence that allergens administered in the airways of mice or humans can recruit and activate lung DCs. However, this activation is not always necessarily direct. Stromal cells like epithelial cells are uniquely positioned at the interface between 'inside versus out,' making them perfect candidates for orchestrating immune responses. Epithelial cells possess microbial-detection mechanisms and express a tightly regulated and specifically localized set of PRRs (**table 1**), which enables them to respond to antigens and allergens like immune cells. An emerging concept is that the epithelial barrier influences the development of antigen-specific immunity, suggesting that the type of immune response is defined by the local tissue microenvironment [7]. It is therefore very likely that antigen recognition by epithelial cells determines the functional properties of tissue-residing DCs, thereby instructing the outcome of antigen-specific immunity. This concept has important implications for the regulation of mucosal homeostasis and the initiation of innate and adaptive immune responses [88]. *In vitro* studies have shown that airway epithelial cells (AECs) exposed to HDM produce CCL2 and CCL20, chemokines attracting immature DCs and their precursors [63, 89]. CCL20 secretion was not dependent on TLR2/4 expression or on the protease activity of allergens, but relied on beta-glucan moieties within HDM [63, 90]. Proteases in pollens, cockroach and HDM were found to disrupt epithelial tight junctions and, in this way, gain access to DCs. In addition, allergens induce the production of inflammatory and instructing pro-Th_2 cytokines by AECs [91-94]. All these data point to the fact that allergen-activated AECs might contribute to instruct DCs and promote Th_2 sensitization. This has been confirmed *in vivo* in a series of experiments using bone marrow chimeric mice lacking TLR4 on either immune cells or radioresistant epithelial cells. The response to HDM was substantially altered when epithelial cells could not sense the endotoxin in the allergen [55, 95]. Interestingly, NF-κB activation was often observed in epithelial cells exposed to allergens and several studies have demonstrated that lung epithelial cell-specific deletion of NF-κB lead to decreased cytokines responses [96-98]. On the opposite, the selective activation of NF-κB in airway epithelial cells was enough to drive allergic airway inflammation [99, 100].

Epithelial cell-derived factors instruct lung DCs to induce Th_2 responses to allergens

NF-κB activation of AECs in response to inhaled allergens, leads to a release of a variety of cytokines (GM-CSF, TSLP, IL-25, IL-33 and IL-1 family members), contributing to DC maturation and driving into a Th_2-activating mode [55, 101] (**Figure 4**). These cytokines are described in more detail below.

GM-CSF

GM-CSF is produced by AECs exposed to allergens [90, 102]. The importance of GM-CSF in Th_2 responses came from data obtained in mice overexpressing GM-CSF in the lung. When exposed to the harmless antigen ovalbumin, mice overexpressing GM-CSF showed a break of inhaled tolerance and developed a strong allergic airway inflammation driven by mature DCs

[103]. Along this line, BMDCs grown in the presence of GM-CSF were found to be very effective at inducing Th_2 priming [16, 104]. Moreover, the administration of HDM in the airways also leads to Th_2 sensitization that was partially mediated by endogenous GM-CSF [105]. In mouse models of HDM-induced asthma, GM-CSF neutralization led to reduced Th_2 responses [106, 107]. GM-CSF is a protypical cytokine used to induce the formation of monocyte-derived DCs from bone marrow progenitors *in vitro*. When GM-CSF cultured DCs are pulsed with protein antigens or real life allergens, and are subsequently transferred to the airways of naïve mice, they induce vigorous Th_2 immunity and asthma develops upon subsequent intranasal challenge with the protein or allergen [16]. However, *in vivo* it is not known yet how crucial GM-CSF is for the formation of moDCS under conditions of inflammation. One recent study observed no reduction of moDC formation in a mouse model of inflammation in GM-CSFR deficient mice [108].

TSLP

Another well-described cytokine affecting DCs, B cells and CD4+ T cells is TSLP. It is produced not only by epithelial cells [55] but also by mast cells and DCs under inflammatory conditions [109, 110]. Various groups have reported correlations between TSLP polymorphisms and/or protein expression levels and the development of asthma or allergic rhinitis [111-113]. Its role in Th_2 responses was shown in a model of asthma in which mice lacking TSLP receptor (TSLPR) were found to have attenuated Th_2 features [114], whereas mice overexpressing TSLP in the lung epithelium had increased asthma features [115]. Recently, TSLP has been shown to break inhaled tolerance by inhibiting the generation of induced allergen-specific regulatory T cells [116]. Moreover, blockade of TSLP using soluble TSLPR *in vitro* led to reduced expression of maturation markers on TSLP-activated DCs. In addition, administration of this soluble TSLPR before sensitization reduced eosinophilia, IgE levels and Th_2 cytokines *in vivo* [117]. TSLP induces Th_2 responses most likely through its capacity to activate DCs to upregulate OX40L, allowing them to expand Th_2 memory cells and enhance the expression of IL-25R (IL-17RB) on these memory cells [118-120]. Overexpression of TSLP in bronchial epithelial cells boosts Th_2 immunity in the lungs [114]. However, in mouse models of asthma, driven by natural allergens, the neutralization of TSLP does not necessarily lead to reduced features of allergy [121, 122]. Despite these doubts on the potential of neutralizing TSLP as a therapeutic strategy, the expression of TSLP is increased in bronchial biopsies and sputum of human asthmatics, particularly in severe disease [123, 124]. Genetic polymorphisms in the promoter region of TSLP are associated with increased risk of asthma [125]. Proteolytic allergens, diesel exhaust particles, and cigarette smoke induce epithelial production of TSLP that causes DC activation [126, 127]. TSLP promotes the growth and differentiation of basophils from the bone marrow [128]. The TSLPR is not only expressed by hematopoietic cells but also by human bronchial ECs. TSLP stimulates the proliferation of bronchial epithelial cells and EC IL-13 production [124]. One further caveat regarding therapeutic targeting of TSLP is that it can contribute to epithelial repair both by inducing secretory leukocyte protease inhibitor (SLPI) and via other mechanisms [124, 129]. Expression of SLPI has the potential to dampen allergic airway inflammation and interfering with TSLP therapeutically might abolish this protective pathway [130].

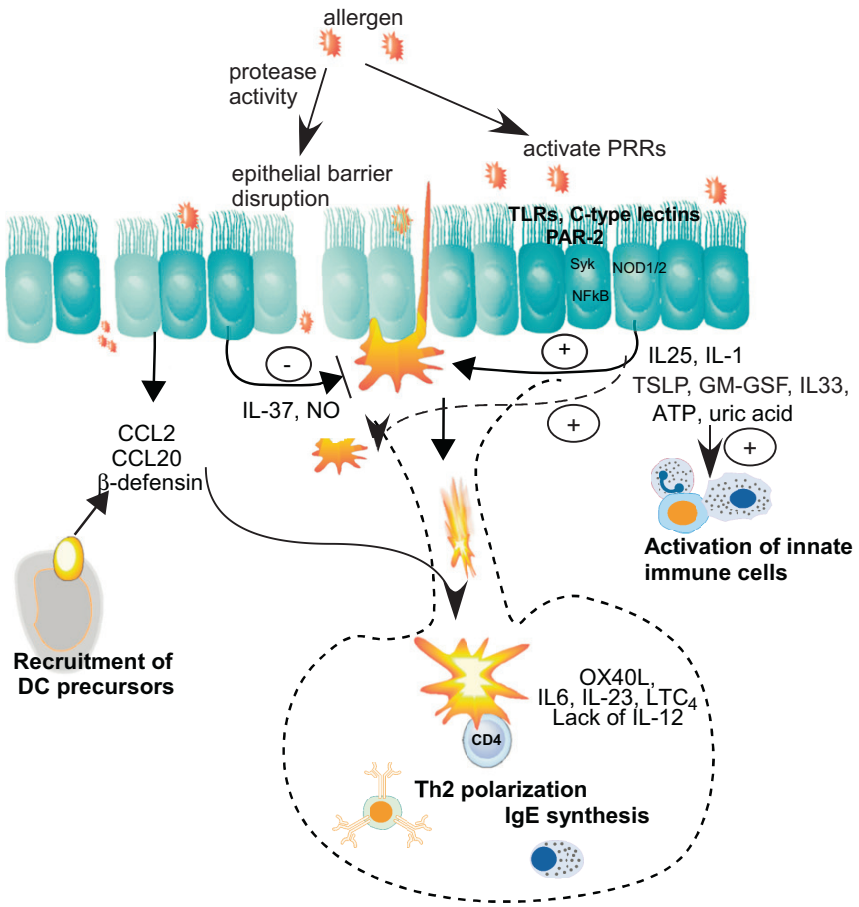

Figure 4: Both lung DCs and epithelial cells express pattern recognition receptors (PRRs) and can be activated directly by allergens. In response to allergens, lung epithelial cells produce chemokines (CCL2, CCL20) that attract immature cDCs and inflammatory monocytes. Activated epithelial cells produce instructing cytokines (like IL-1, GM-CSF, and TSLP) and danger signals (ATP, uric acid), favoring DC maturation and their migration to the MLN, where they will induce Th_2 responses. These cytokines and danger signals also recruit and activate innate immune cells, such as basophils, mast cells and ILCs. Basophils secrete IL-4 and type 2 innate lymphoid cells make IL-5 in response to IL-33 release by epithelial cells, providing a pro-Th_2 environment. Some epithelial-derived cytokines like IL-37 are able to suppress immune responses by inhibiting DC activation.

IL-25

IL-25 (also known as IL-17E) has initially been reported as a Th_2 cell-derived cytokine [131]. However, it can also be produced by basophils [132] and epithelial cells in response to allergens and respiratory viruses [133, 134]. The proteolytic enzyme MMP7 from bronchial ECs is necessary for optimal production of IL-25 [135]. The production of IL-25 by epithelial cells is suppressed by IL-22 [136]. IL-25 acts on the IL-25R that consists of the IL17RB and IL25R subunits and signals via the adaptor Act1. Although IL-25 promotes Th_2 immunity in the lung [132, 137], its potential to activate DCs remains unclear. Epithelial-derived IL-25 induces Jagged-1 expression on DCs and leads to Th_2 responses in the lung of RSV-infected mice [138]. Furthermore, IL-25 induces IL-9 production by Th_9 cells, via the IL-17RB subunit [139]. When administered via the airways, it acts directly on pre-ILC2 to induce their expansion and activation [140]. Recently, it was shown that IL-25 also expands a population of granulocytic

myeloid cells that produce IL-5 and IL-13 and contribute to lung pathology in mice and humans [141]. Epithelial IL-25 also acts directly on fibroblasts and endothelial cells to promote airway remodeling and angiogenesis and boosts production of TSLP and IL-33 [142]. Together, these studies imply that IL-25 and IL-17RB may be effective therapeutic targets, as they are upstream of Th_2, Th_9 and ILC2 immunity and epithelial activation. Besides, the number of IL-25-expressing and IL-25R-expressing cells is increased in biopsies of asthmatic patients [143, 144]. In mice, overexpression of IL-25 in lung epithelial cells led to increased allergic inflammation [133], probably owing to the capacity of IL-25 to activate DCs in driving Th_2 responses [134]. Injection of neutralizing anti-IL-25 antibodies strongly reduced allergic airway inflammation and airway remodelling [133, 145].

IL-1RI binding family members

The IL-1 family consists of several members and the family is still increasing. IL-1α, IL-1β and IL-1Ra both bind to IL-1 receptor 1 (IL-1RI), which is present on almost all cell types, including structural cells. IL-1β is released from cultured AECs following protease allergen exposure and enhances the release of the DC-attracting chemokine CCL20, and of the DC maturation cytokines TSLP and GM-CSF [146, 147]. Remarkably mice deficient of IL-1 α/β showed identical AHR as control mice in an asthma model with OVA alum sensitization. However, in a milder model using repetitive OVA injections intraperitoneally, mice deficient of IL-1 α/β showed a reduced AHR. In addition, mice deficient for IL-1RA (natural antagonist) showed increased influx of DCs to the lung, AHR and levels of specific IgE and Th_2 responses [148]. Mice lacking IL-1RI had reduced features of asthma in the mild model, but not when alum adjuvant was used to sensitize the mice [149]. Monocytes and dendritic cells were the main inflammatory cell types secreting IL-1β [150]. IL-1RI signalling involves the adaptor molecule MyD88, also found downstream of several TLRs. Interestingly, HDM induced Th_2 responses in the lung were found strongly reduced in MyD88-/- mice [56]. In this study, DC migration to the LNs was altered in MyD88-/- but not in TLR4-/- mice, suggesting that some aspects of DC biology might be controlled by IL-1 family members signalling through MyD88. Recently, more attention has been put on trying to elucidate the role of other IL-1 family members. As such, IL-37 was reported to be different from other members of the family since it was able to suppress inflammatory responses. When expressed in lung epithelial cells or in macrophages, IL-37 decreased the production of pro-inflammatory cytokines, such as IL-1α, TNFα and IL-6 by these cells [159]. Little information is available about another member called IL-1F9. Elevated levels of this cytokine have been detected in AECs of allergen-challenged mice and it was shown to promote inflammation [160]. Whether this epithelial-derived cytokine is able to instruct lung DCs to induce Th_2 responses to inhaled allergens remains to be elucidated.

IL-33

DCs have been shown to express ST2, the receptor for the IL-1 family member IL-33. This cytokine is expressed in AECs where it is constitutively stored in the nuclei. Its location is unexpected and how it is released in the extracellular environment remains unclear. Although it has been postulated that IL-33 might act as an alarmin in case of allergen exposure [151]. Besides DCs other inflammatory cells express ST2, like eosinophils, basophils, mast cells and type 2 innate lymphoid cells (ILC2). DCs exposed to IL-33 increased their expression of

maturation markers, such as CD40, CD80 and OX40L and became very potent at inducing Th_2 responses [152, 153]. Intratracheal administration of IL-33 induces eosinophilia in the lung and increased immunoglobulin serum levels [152]. Moreover, human eosinophils were shown to become activated by signalling of the ST2 receptor *in vitro* [154]. In addition, in a mild asthma model treatment of mice with an antibody against IL-33, features of airway inflammation were inhibited [155]. IL-33 was found to be elevated in biopsies from asthmatics compared to control subjects [156]. This cytokine is also released by lung epithelial cells upon HDM challenge and levels were found to correlate with AHR [55, 157]. Recently IL-33 was found to be cleaved by caspase-1 just like its family members IL-1β and IL-18. However, the cleaved protein was inactive and not able to bind to the ST2 receptor and induce signalling, unlike the intact IL-33 [158].

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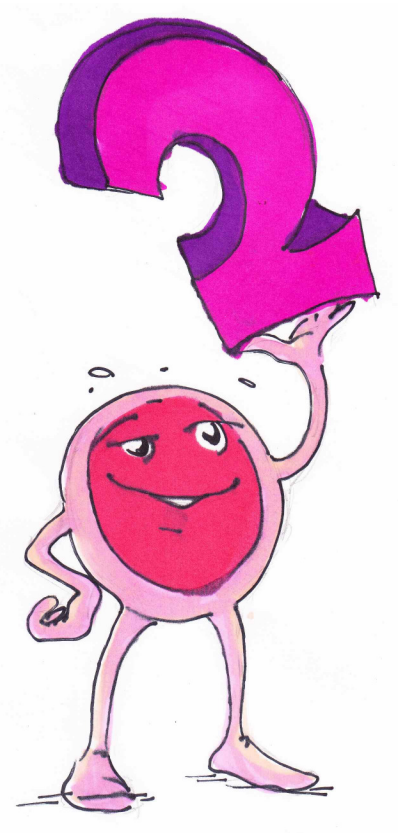

Aims and outline of the thesis

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As described in the introduction, DCs are the main antigen presenting cells responsible for immune activation in the lung. DCs can react directly to variety of antigen and allergens. At the outset of this thesis, we had three questions that also currently dominate the field of lung DC biology:

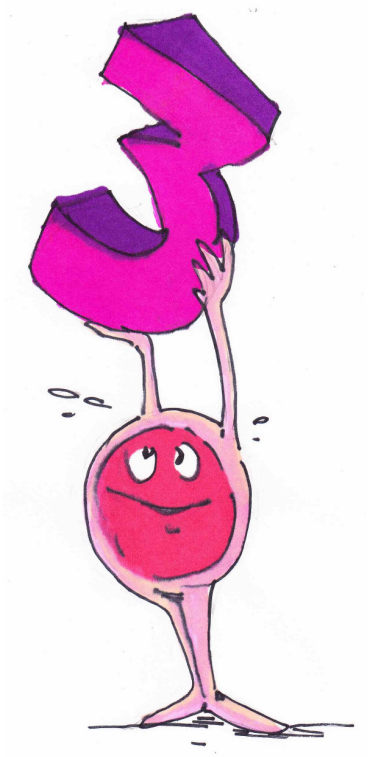
1/ Which subsets are performing the task of antigen presentation in the lung?

2/ Which signals are required to get proper activation of DCs in the lungs in response to foreign matter introduction?

3/ Can we exploit DCs to design better therapies for inflammatory lung disease?

Although the primary focus of this thesis is on asthma, we also addressed the function of the lung DCs that surround the lung capillaries, one of the last DC subsets on which very little is known. In **chapter 3**, we investigated if and how large particulate antigens that were injected in the bloodstream are presented by DCs in the lungs. We describe a model using sepharose beads to investigate antigen sampling and presentation by blood vessel lining DCs. We found that inflammatory mo-DCs are presenting the antigen to T cells in the MLN. In **chapter 4** we describe the potential therapeutic application of the immunomodulary compound, ursodeoxycholic acid (UDCA) in a model of experimental asthma. We found that UDCA is reducing allergic lung inflammation by acting on the farnesoid X-receptor on DCs.

While investigating the role of endogenous danger signals in allergic asthma to better understand the activating signals of lung DCs, we show in **chapter 5** that uric acid acts on lung DCs during sensitization to allergens and depends on TLR4 signalling on structural cells. Trying to elucidate the downstream effects of IL-1 in allergic asthma, we found that IL-1RI signalling on radioresistant stromal cells plays a key role in the sensitization in allergic asthma. As presented in **chapter 6** we found that IL-1α, not IL-1β, was important in allergic asthma leading to release of GM-CSF and IL-33 by AECs, instructing DCs to induce a Th_2 immune response. In **chapter 7** we discuss some recent findings relevant to the published data and touch upon the clinical applicability of our findings.



The lung vascular filter as a site of immune induction for T cell responses to large embolic antigen

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In this chapter we have set up a model to study large embolic antigens. Dendritic cells were found to surround the blood vessels in the lung. Conventional DCs recruit monocytes to the site of inflammation by releasing MCP-1. We found that recruited monocyte derived-DCs are capable to present these antigens to primary T cells in the draining lymph node.

The lung vascular filter as a site of immune induction for T cell responses to large embolic antigen

Abstract

The bloodstream is an important route of dissemination of invading pathogens. Most of the small bloodborne pathogens, like bacteria or viruses, are filtered by the spleen or liver sinusoids and presented to the immune system by dendritic cells (DCs) that probe these filters for the presence of foreign antigen (Ag). However, larger pathogens, like helminths or infectious emboli, that exceed 20 µm are mostly trapped in the vasculature of the lung. To determine if Ag trapped here can be presented to cells of the immune system, we used a model of venous embolism of large particulate Ag (in the form of ovalbumin [OVA]-coated Sepharose beads) in the lung vascular bed. We found that large Ags were presented and cross-presented to CD4 and CD8 T cells in the mediastinal lymph nodes (LNs) but not in the spleen or liverdraining LNs. Dividing T cells returned to the lungs, and a short-lived infiltrate, consisting of T cells and DCs, formed around trapped Ag. This infiltrate was increased when the Toll-like receptor 4 was stimulated and full DC maturation was induced by CD40 triggering. Under these conditions, OVA-specific cytotoxic T lymphocyte responses, as well as humoral immunity, were induced. The T cell response to embolic Ag was severely reduced in mice depleted of $CD11c^{hi}$ cells or $Ly6C/G^{+}$ cells but restored upon adoptive transfer of $Ly6C^{hi}$ monocytes. We conclude that the lung vascular filter represents a largely unexplored site of immune induction that traps large bloodborne Ags for presentation by monocyte-derived DCs.

Introduction

DCs are the most important APCs for mounting a primary immune response to foreign antigens (Ags) that invade the various barriers of the body such as the skin, gastrointestinal, and respiratory mucosae (Banchereau and Steinman, 1998). DCs have been well studied and their function is to take up Ag across the lining barrier, integrate signals on the pathogenicity of the Ag, and migrate via the afferent lymph to the regional draining LNs. In the T cell area of these draining LNs, DCs induce a tailor-made immune response that is optimal to clear the pathogen in the best possible way while avoiding damage to self (Banchereau and Steinman, 1998). Another portal of entry, but also a portal of dissemination of invading pathogens, is the bloodstream. Pathogens that reach the bloodstream, either through direct puncture of the skin or invasiveness through mucosal surfaces such as the nasopharynx or lung, are easily carried throughout the body. It is most often assumed that these Ags will be filtered by the splenic microarchitecture, particularly the splenic marginal zone, and subsequently presented by splenic DCs and/or macrophages to naive T cells for induction of a primary protective immune response (De Smedt et al., 1996; Morón et al., 2002). Some bloodborne pathogens might also be filtered and presented in the bone marrow (Feuerer et al., 2003). For pathogens that replicate and invade the bloodstream via the gastrointestinal tract, the portal circulation can lead to filtering in the liver blood sinusoids and Ag presentation by DCs in liver draining LNs (Kudo et al., 1997). Another, often neglected mechanism might exist in the lungs to filter bloodborne pathogens. The pulmonary vascular system with its small diameter arterioles (20–

500-µm diameter) and capillaries (<10-µm diameter) forms an extensive meshwork that receives the complete cardiac output of blood (for comparison, the spleen receives only 5% of cardiac output or 200–300 ml/min). Large particles that circulate in the bloodstream (called emboli in the medical literature) and exceed the diameter of the small pulmonary vessels are very efficiently trapped by this system. It is currently unknown whether Ags or pathogens that clog the lung vascular system would be presented to the immune system in a way that leads to protective immunity, which is analogous to the splenic or liver filter system. The lung vascular bed is situated in the lung interstitium, where a well-developed network of interstitial DCs and macrophages is present (GeurtsvanKessel and Lambrecht, 2008). However, APCs of the lung interstitium have generally been regarded as sessile cells that only stimulate already primed T cells, for example, during pulmonary delayed-type hypersensitivity reactions and granuloma formation (Holt et al., 1988; Gong et al., 1994; Iyonaga et al.,2002; Tsuchiya et al., 2002). DCs are also found to line the intima and adventitia of larger (pulmonary) vessel walls and, therefore, could probe the luminal contents for the presence of Ags, although it is unclear to what Ags this sampling system would react (Millonig et al., 2001; Perros et al., 2007; Choi et al., 2009). The purpose of this study was to determine whether the lung vascular system allows Ag sampling and, thus, acts as a site of immune induction for T cell responses after i.v. injection of particulate Ag. For this, we injected large Sepharose beads coupled or not to ovalbumin (OVA). Because of their size (± 40–150 µm), i.v.-injected beads are specifically retained in the vascular bed of the lung. Our studies revealed a hitherto unexplored potential of $CD11c^+$ DCs and their immediate monocytic precursors to sample the lung vascular compartment for trapped Ag, leading to Ag presentation in lung draining LNs and generation of effector responses to OVA.

Results

Embolic large particulate Ag is presented and cross-presented exclusively in the mediastinal LNs (MLNs) draining the lung

We i.v. injected large Sepharose beads coupled or not to $OVA_{323-339}$ peptide (coded, respectively as OVA beads [OBs] and uncoated beads [UBs]), the immunodominant MHCII-restricted peptide of OVA. To allow detection of the precise site of primary T cell activation and division, 2 d earlier, mice received a cohort of CFSE-labeled CD4+ OVA TCR transgenic (Tg) DO11.10 T cells (**Fig. 1 A**), recognizing the $OVA_{323-339}$ peptide in the context of $I\text{-}A^d$ (MHCII). The precise localization and degree of T cell proliferation, as measured by sequential halving of CFSE intensity with each round of T cell division, was measured over time in different anatomical compartments, including the lung, draining MLN, peripheral nondraining LN, and spleen, up until 16 d after bead injection. Proliferation of naive OVA-specific T cells first occurred exclusively in draining MLNs of the lung 2 d after injection of OBs but not after injection of UBs (**Fig. 1 B**). Despite the i.v. injection of Ag, no divisions were observed in spleen or nondraining peripheral LNs (PLNs), illustrating that free or particle-bound Ag did not leak from trapped Ag or pass beyond the lung capillary filter. More specifically, we also tested whether primary divisions were found in the liver draining LNs yet found no evidence for this (**Fig. S1**). When we focused on the lung interstitium itself, which was obtained by enzymatic digestion of a lung lobe, we could not detect divided T cells 2 d after injection of beads (**Fig. 1 C**). When the T cell response was followed over time (**Fig. 1 B**), it became evident that by day 4

after injection of OBs, divided CD4+ T cells appeared in the nondraining nodes as well as the spleen. These T cells had divided at least three to four times and expressed high levels of CD44 while having down-regulated the early activation marker CD69, which is consistent with an activated phenotype, as previously reported (Lambrecht et al., 2000; unpublished data). Strikingly, these divided cells could also be traced back to the lung interstitium (by flow cytometry; **Fig. 1 C**), where they were found surrounding the injected OBs (by confocal imaging and immunostaining for DO11.10 TCR; **Fig. 1 D**). Some OVA-specific DO11.10 T cells were seen in immediate proximity of lung vessels in the vicinity of OBs, suggesting a specific recruitment mechanism at this site. When the immune response was evaluated even later (days 8 and 16), it was clear that divided $CD4^+$ T cells could no longer be traced back in the LNs, spleen, or lung, suggesting that they were deleted or their numbers had declined to a level below the threshold of detection by flow cytometry (**Fig. 1 B**). Although these findings certainly suggested that there is a mechanism of immune induction for Ag trapped in the lung vasculature, these experiments were not conclusive as to whether Ag would also be processed, as beads were coated with preprocessed OVA peptide. We therefore also studied whether whole OVA protein coupled to Sepharose beads would be processed and presented to CD4 and cross-presented to CD8 T cells. We therefore turned to the C57BL/6 background, in which both CD4 (OT-II) and CD8 (OT-I) TCR Tg mice are available (Barnden et al., 1998). For this, C57BL/6 mice received MHCI-restricted OT-I or MHCII-restricted OT-II OVA TCR Tg T cells. In both circumstances, we observed T cell divisions in the MLNs 4 d after injection of OBs (**Fig. 1, E and F**). Again, injection of OBs did not lead to T cell divisions outside the draining area of the lung (unpublished data). In the lungs, we followed the size of the inflammatory lesions surrounding OBs or UBs. By day 4 of the response, the inflammatory lesions (as measured by the mean surface area of the inflammation minus the surface area of the bead divided by the bead surface area [see Materials and methods]) was greatly enhanced in mice receiving OBs compared with UBs. However by days 8–16, all inflammation surrounding the beads had disappeared, illustrating the transient nature of lung inflammatory lesions (**Fig. 1 G**). Together, these data suggested the presence of an active immune surveillance mechanism in the lung vascular bed that was induced in the mediastinal nodes.

Monocyte-derived DCs accumulate around particulate Ag trapped in the lung vasculature

We next addressed by which mechanism trapped Ag would be presented in the lung vascular bed. First, we studied the distribution of injected beads after i.v. injection. Injected beads were found exclusively in the lung vascular bed (**Fig. 2 A**) but not in the draining MLN, nondraining LN, or spleen (not depicted). As there was Ag presentation in the MLN, despite absence of macroscopic beads at this site, we reasoned that a migratory APC population might carry antigenic cargo to the node. As soon as 6 h after injection of UBs or OBs, we detected a population of $CD11c^+$ cells around the injected beads, irrespective of whether beads were coated with OVA peptide (**Fig. 2 A**, days 1–16) or not (not depicted). In addition, MHC class II^+ cells started to appear around these beads around this time period. Double staining revealed these cells to be $CD11c^+$ and MHC class II^+ double positive, strongly suggesting they were DCs (**Fig. 2 A**). When followed over time, the number of $MHCII^+CD11c^+$ cells was increased at day 4 after injection of OBs compared with injection of UBs, a time point when inflammatory lesions and accumulation of $CD4^+$ T cells were also maximal (**Fig. 2 B**). Later in the response, $CD11c^+$

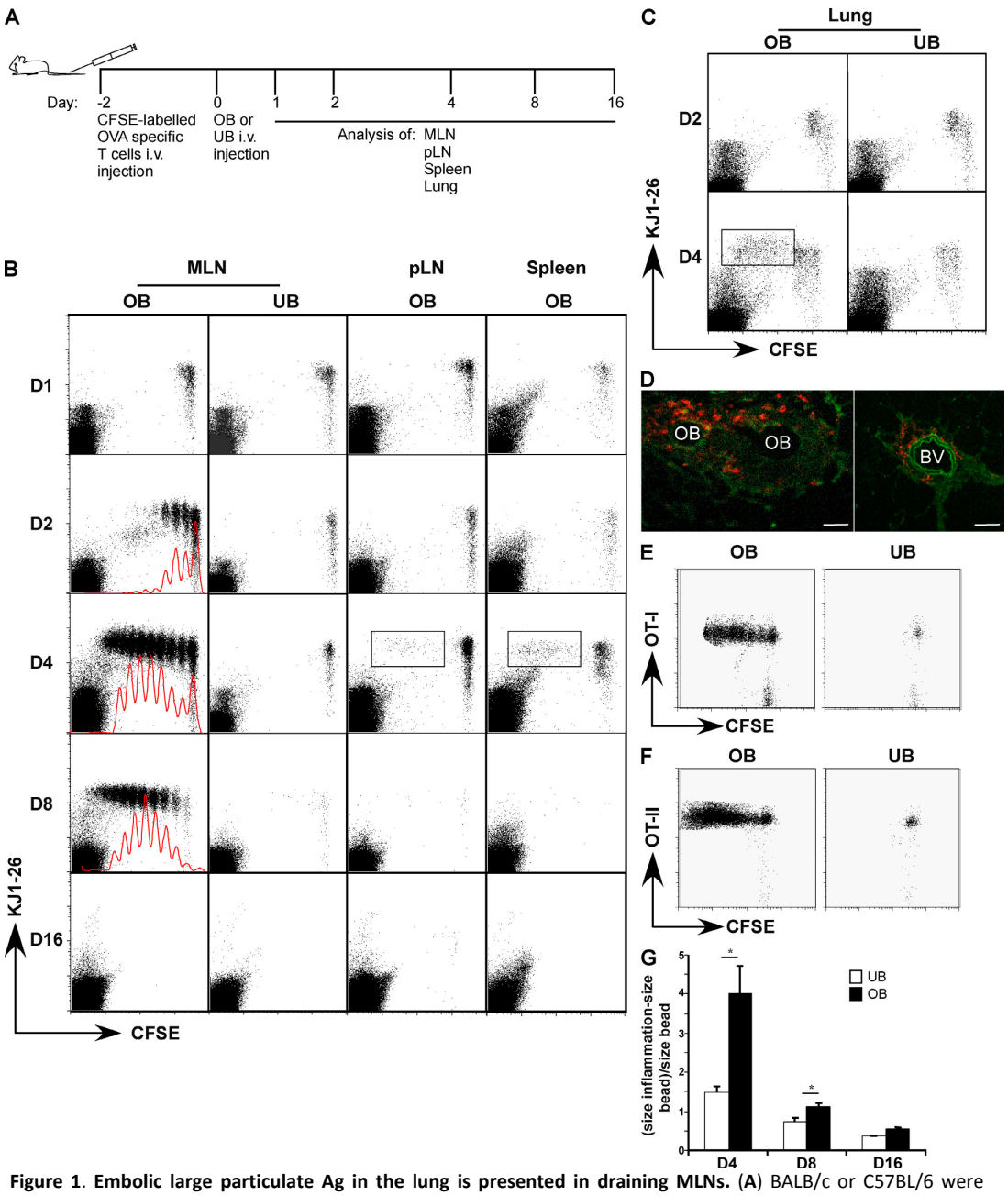

Figure 1. **Embolic large particulate Ag in the lung is presented in draining MLNs.** (**A**) BALB/c or C57BL/6 were injected with OVA-specific CFSE-labeled TCR Tg T cells 2 d before i.v. injection of particulate Ag (OVA 323-339 peptide, LPS-free OVA, or control glycine). At 1, 2, 4, 8, and 16 d after injection of particulate Ag, mice were sacrificed and MLNs, PLNs, spleen, and lungs were analyzed for a T cells response. This experiment was performed three times with four mice at each time point. (**B**) Response of OVA-specific CFSE-labeled $CD4^+$ DO11.10 T cells after injection of OBs or UBs at indicated days. MLNs, PLNs, and spleens were analyzed for the occurrence of OVA T cell division (CFSE profile, x axis) in OVA-specific $KJ1.26^+$ T cells (y axis). Recirculating divided T cells are indicated in rectangles. FACS plots represent a single animal of a group of five at each day point. The experiment was performed three times. (**C**) Division profile (CFSE, x axis) of $KJ1\text{-}26^+$ $CD4^+$ DO11.10 T cells (y axis) in the lung at 2 and 4 d after injection of OB or UB. Recirculating divided T cells are indicated in rectangles. FACS plots represent a single animal of a group of five at each day point. The experiment was performed three times. (**D**) Confocal imaging of frozen section of lungs stained with the OVA-specific TCR-specific marker KJ1-26 (red). At day 4 after injection of OBs, OVA-specific T cells could be found in close proximity to blood vessels (BV) and around the OB. Bars, 40 µm. (**E**) Whole particulate Ag is cross-presented in draining MLNs of the lung. At day - 2, OVA-specific Tg $CD8^+$ CFSE-labeled OT-I

were injected i.v., and at day 0, OBs (LPS free) or UBs were injected i.v. At day 4, MLNs were gated on CD8 T cells and analyzed for division CFSE profile (x axis) of Vβ 5.1/5.2–PE-positive OVA-specific T cells (y axis). This experiment was performed three times with four mice per group. (**F**) Whole particulate Ag is presented in draining MLNs of the lung. Division CFSE profiles (x axis) of CD4 + Vβ 5.1/5.2–PE-positive (y axis) OT-II OVA-specific T cells in MLNs, 4 d after OB injection. Cells were gated for CD4 positivity. This experiment was performed three times with four mice per group. (**G**) Size of inflammation surrounding UBs or OBs in the lung as measured by image analysis measuring at days 4, 8, and 16 after injection of UBs (white bars) and OBs (black bars). Groups consisted of five mice at each day point. The experiment was performed three times. Mean ± SEM of the group is depicted. *, $P < 0.05$.

DCs were still present around OBs but, gradually, their numbers decreased. To check for the presence of myeloid cells (like monocytes, neutrophils, and some DC subsets), we also stained for the presence of $CD11b^+$ cells. We found $CD11b^+$ cells to be abundantly present around OBs (**Fig. 2 A**, bottom). The CD11c integrin is expressed on different cell types in the mouse lung but is mainly restricted to $CD11b^-$ and $CD11b^+$ conventional lung DCs, inflammatory type $CD11b^+$ DCs, and $CD11b^+$ alveolar macrophage in the lung (de Heer et al., 2004; van Rijt et al., 2004; Vermaelen and Pauwels, 2004; Sung et al., 2006; GeurtsvanKessel et al., 2008). Lung plasmacytoid DCs (pDCs) are characterized by intermediate expression of CD11c and expression of Gr1 (Ly6G/C), B220, and the specific marker BST2 (recognized by the mAb 120G8; Asselin-Paturel et al., 2003; de Heer et al., 2004, 2005). To analyze which subset of $CD11c^+$ DC was recruited to particulate Ag, frozen sections were stained with CD11c and Gr1 (recognizing Ly6C/G) or B220. Remarkably, $CD11c^+$ cells surrounding beads were $Gr1^+$ but lacked expression of the B220 marker, which is normally expressed on the surface of pDCs (**Fig. S2**). We also stained for pDCs by using the pDC-specific antibody 120G8, and this staining confirmed that $Gr1^+$ $CD11c^+$ DCs were not pDCs (unpublished data). We did not observe significant amounts of CD103, which is found on a subset of $CD11b^-$ lung DCs (unpublished data). The expression of Gr1 (Ly6G/C) on lung $CD11c^+$ cells surrounding beads was unexpected, as this marker is classically not found on lung DCs in steady state (GeurtsvanKessel and Lambrecht, 2008; GeurtsvanKessel et al., 2008). Gr1 also recognizes the Ly6C Ag, expressed on a subset of monocyte-derived inflammatory DCs (Randolph et al., 1999; Geissmann et al., 2003; Sunderkötter et al., 2004; León et al., 2007; León and Ardavín, 2008). In the mouse, circulating monocytes can be discriminated into $Ly6C^+$ $CX3CR1^{int}$ $CD11b^+$ monocytes and $Ly6C^+$ $CX3CR1^{hi}$ $CD11b^+$ monocytes (Geissmann et al., 2003). Several groups have now shown that both types of monocytes are immediate circulating precursors for lung DCs but not steady-state lymphoid tissue conventional DCs (Geissmann et al., 2003; Landsman et al., 2007; Varol et al., 2007; Jakubzick et al., 2008). To test the presence and differentiation of monocytes around injected beads at earlier time points, we injected $CX3CR^{GFP}$ mice with OBs (Jung et al., 2000). In these $CX3CR^{GFP}$ mice, all monocytes (and some T cells and NK cells) can be identified by GFP positivity and precisely localized in tissues using confocal imaging. As shown in **Fig. 2 C**, 6 h after injection of OBs, CX3CR-GFP^+ cells were seen around injected beads. At the same time, $Ly6C^+$ and $CD11c^+$ cells were seen. The majority of cells were triple positive. It is of note that few cells were $CX3CR1^+Ly6C^-$, signifying resident blood monocytes, although some cells expressed CX3CR1 and Ly6C without CD11c, representing recruited inflammatory monocytes (Auffray et al., 2007).

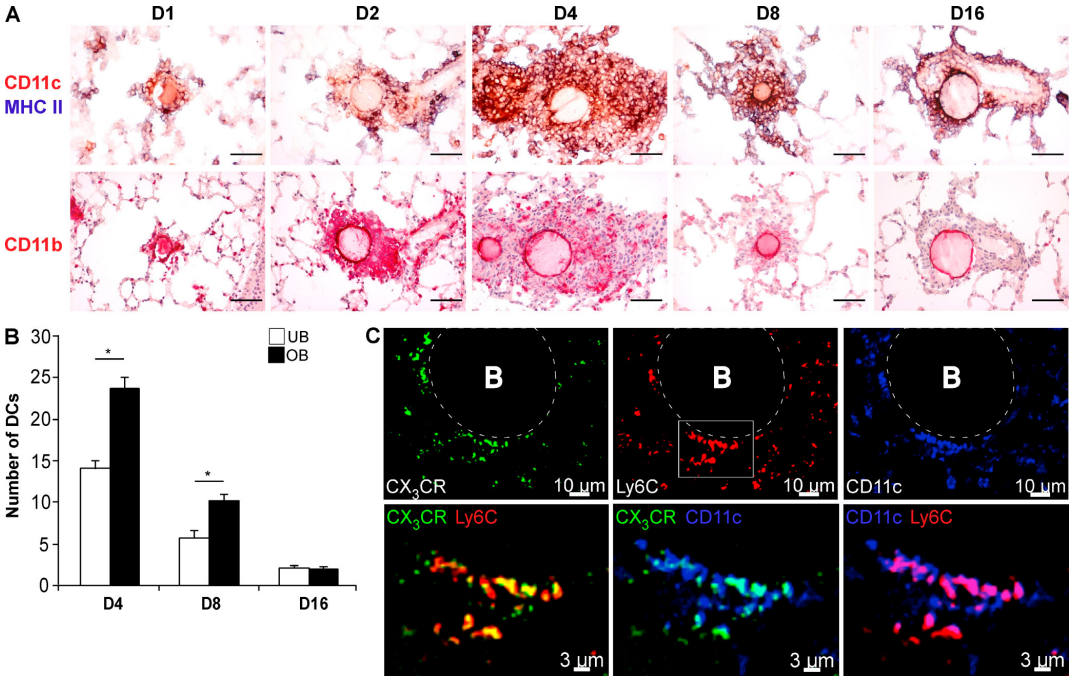

Figure 2. DCs and their monocytic precursors surround trapped particulate Ag. (**A**) 6-µm lung sections at 1, 2, 4, 8, and 16 d after injection of OBs were double stained for CD11c and MHC class II expression to identify the appearance of DCs around the particulate Ag. Furthermore, myeloid cells surrounding OB were stained with an anti-CD11b antibody on a consecutive slide. UBs are not shown in A but are summarized under B. This experiment was performed three times with four mice per group. Bars, 40 µm. (**B**) Absolute number of DCs surrounding OB and UB (double stained for $CD11c^+$ and $MHCII^+$) were counted for n = 4 mice per day per group. Results are shown as mean of $CD11c^+$ $MHCII^+$ DCs of 30 separate lesions surrounding beads ± SEM. *, $P < 0.05$. This experiment was performed twice. (**C**) Presence of monocytic markers on $CD11c^+$ cells surrounding OBs. For this purpose, C57BL/6 CX3CR-GFP (green) Tg mice received OBs and OTII T cells. Lungs were sampled 6 h after OB i.v. injection. Slides were additionally stained for Ly-6C (red) and CD11c (blue). Beads are marked by dashed circles and a capital B. Overlays of a frame depicted in the top middle (rectangle) are shown in the bottom. This experiment was performed twice.

Monocyte-derived DCs are necessary and sufficient for presentation of particulate Ag trapped in the lung vasculature

These findings of Ag presentation in the MLN draining the lungs and accumulation of $Ly6C^+CD11c^+$ cells around injected beads suggested that DCs were the major APC population presenting bead-associated intravascular Ag to T cells. To address the precise contribution of $CD11c^+$ DCs, we used mice expressing the human diphtheria toxin (DT) receptor (DTR) under the control of the murine CD11c promotor, allowing the DT-induced conditional depletion of all conventional $CD11c^+$ DCs but leaving pDCs largely unaffected (Jung et al., 2002; Sapoznikov et al., 2007). We administered DT i.p. at the time of OB injection and followed the division of CFSE-labeled DO11.10 T cells. As seen in **Fig. 3 A**, T cell proliferation at day 4 of the response in the MLNs of CD11c- DTR Tg mice given DT was dramatically reduced to the level seen in mice given UBs. However, non-Tg littermate control mice given DT (or CD11c-DTR Tg mice given PBS [unpublished data]) still mounted a normal response to bead injection. The reduction of T cell proliferation in DC-depleted mice was accompanied by a severe reduction in the size of the inflammatory lesions surrounding the OBs at day 4 after injection (unpublished data). To study if DT treatment depleted local CD11c+ cells around injected beads, we performed

immunostaining for CX3CR1, Ly6C, and CD11c. Whereas in DTR-Tg mice given PBS treatment there was a clear recruitment of triple-positive cells 24 h after injection of OBs, these cells were severely reduced by DT treatment. Very strikingly, the depletion of $CD11c^{hi}$ cells using DT also led to a reduction in $CX3CR^{+}Ly6C^{+}CD11c^{+}$ inflammatory monocytes (**Fig. 3 B**). As the depletion of these monocytes in CD11cDTR Tg mice has never been demonstrated before and as these cells lack expression of CD11c, whose promotor is driving DTR expression, we hypothesized that DT treatment led to a reduction of inflammatory monocytes indirectly. It has been shown that $CD11c^{+}$ cells of the lungs are a predominant source of chemokines for recruitment of other inflammatory cell types (Beaty et al., 2007). In support of this hypothesis, we performed stainings for monocyte chemotactic protein 1 (MCP-1, also known as CCL2), the major chemokine recruiting $CCR2^{hi}$ inflammatory monocytes (**Fig. 3 C**). In PBS-treated DTR-Tg mice given OBs, there was a strong signal for MCP-1 colocalizing with $DAPI^{+}CD11c^{+}$ cells and a clear MCP-1 signal localized to the $DAPI^{neg}$ extracellular space. However in DT-treated DTR-Tg mice, both CD11c associated and extracellular MCP-1 staining was abolished. This data suggested that the effect of DT treatment on Ag presentation of bead-associated Ag could be the result of direct effects on resident DCs or to indirect effects on recruited monocytes that subsequently differentiate to inflammatory type DCs. To further study the contribution of inflammatory DCs in presentation of large embolic Ag, we sorted $Ly6C^{+}CD11b^{+}CD11c^{+}$ inflammatory DCs from the MLN and lungs 24 h after injection of OB or UB (**Fig. 3 D**). Whereas lung-derived inflammatory DCs did not present the OVA to CD4 T cells, MLN derived inflammatory DCs readily did. Similar experiments using CD8 OVA-specific T cells as a read out demonstrated that both MLN and lung inflammatory DCs presented to CD8 T cells (**Fig. S3**). In contrast, autofluorescent $F4/80^{+}CD11c^{+}$ alveolar macrophages did not present the Ag to CD4 or CD8 T cells (**Fig. 3 D and Fig. S3**). Cells with this phenotype were not seen to migrate to the MLN. To more directly address the functional role of $Ly6C^{+}$ monocytes and their offspring in causing T cell proliferation after i.v. injection of embolic Ag, we performed depletion experiments in which monocytes were depleted using the Gr1 (Ly6C/G) antibody (Jakubzick et al., 2008). Single injection treatment with this antibody did not affect the number of lung-resident DC subsets (unpublished data). At day 4, mediastinal T cell division was severely reduced in Gr1- treated mice, compared with isotype-treated mice (**Fig. 3 E**), almost down to the level of mice injected with UBs (not depicted). The formation of inflammatory lesions around injected OB was also significantly suppressed and delayed until day 8 after injection of beads in mice depleted of $Gr1^{+}$ cells compared with the control isotype-treated mice, which had the maximum inflammatory lesions at day 4 (**Fig. S4 A**). However, by day 16, all inflammatory lesions had cleared, even in Gr1-treated mice. The Gr1 antibody has also been used to deplete pDCs from the lungs of mice (de Heer et al., 2004; Smit et al., 2006). To address the specific contribution of pDCs in this response, we also treated mice with the depleting antibody 120G8, recognizing the more pDC-restricted Ag bone marrow-stromal Ag-2 (Asselin-Paturel et al., 2003). The treatment with 120G8 around injection of OBs did not affect the degree of T cell proliferation in draining MLNs at day 4 of the response, compared with isotype-treated mice (**Fig. S4 B**), nor did it affect the size of inflammatory lesions (not depicted). As these experiments in $CD11c^{+}$-depleted and Gr1-treated mice clearly demonstrated that these cells were necessary for immune induction in the lung capillary filter, we next questioned if $Ly6C^{+}$ monocytes would also be sufficient for inducing an immune

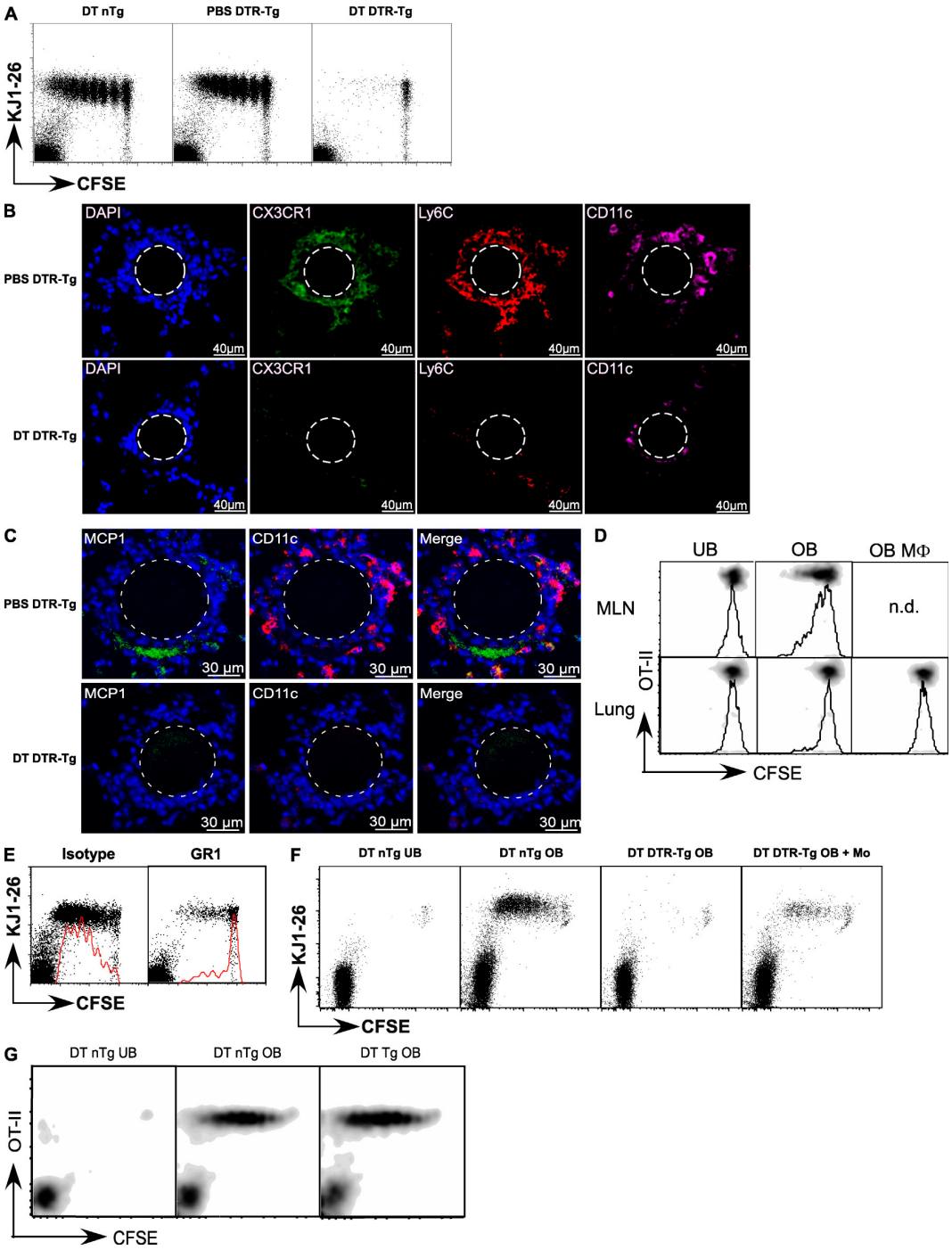

Figure 3. Monocyte-derived CD11c + DCs are essential and sufficient for priming of naive CD4$^+$ in lung draining MLNs in response to Ag trapped in the lung vasculature. (**A**) Effect of DT treatment in CD11c-DTR Tg mice on Ag presentation of large embolic Ag. At day 4 after injection of OB, hardly any division in CFSE-labeled DO11.10 OVA-specific CD4$^+$ T cells could be found in draining lung MLNs in DT-treated DTR-Tg mice, whereas strong divisions were seen at that same time point in non-Tg littermates mice receiving OB and DT or DTR-Tg mice receiving OB and PBS as a control treatment. (**B**) Effect of DT treatment on presence of monocytes and DCs around beads. 24 h after DT treatment, fewer CX3CR1$^+$ Ly6C$^+$ monocytes and CX3CR1$^+$ Ly6C$^+$ CD11c$^+$ inflammatory DCs were found around injected OB in CD11c-DTR-Tg mice compared with PBS treatment. Beads are marked by dashed circles. (**C**) Effect of

DT treatment on monocyte specific chemokines. $CD11c^+$ cells were depleted with DT injection on day 0 in CD11c DTR-Tg mice. 24 h after DT treatment and OB injection, no MCP-1 was found around injected beads, whereas in PBS-treated Tg mice, MCP-1 was found co-localizing with CD11c and in the extracellular space not containing DAPI + nuclei. Beads are marked by dashed circles. (**D**) DCs and macrophages were sorted from MLN and lung from mice injected with UB or OB. These APCs were subsequently put in co-culture with CFSE-labeled OT-II cells for 4 d. Autofluorescent alveolar macrophages were not detected (n.d.) in the MLNs. (**E**) Effect of depletion of Ly6C/G cells using Gr1 antibody. CFSE-labeled OVA-specific $CD4^+$ DO11.10 division profile in draining MLNs in mice treated with anti-Gr1 or isotype i.p. at day 0 and injected with OB, measured at day 4. (**F**) $Ly6C^{hi}$ monocytes restore T cell division in CD11c-depleted mice. CD11c/DTR-Tg mice or non-Tg littermates (nTg) received CFSE-specific OVA-specific $CD4^+$ T cells at day -2, with DT at day 0, with either UB (nTg) at day 0 or OB (nTg) at day 0, or OB at day 0 (DTR-Tg mice) with or without 3.5×10^5 bone marrow–purified (>99%) $Ly6C^{hi}$ $CD11b^{hi}$ monocytes. $CD4^+$ OVA-specific T cell divisions in these groups are shown in draining MLNs at day 4 after UB or OB injection. DTR-Tg mice depleted of $CD11c^+$ by DT show reconstitution of divisions by monocyte i.v. injection at day 0. (**G**) Depletion of $langerin^+$ lung DCs, using DT injection, in langerin-DTR mice showed no effect on OVA-specific T cell proliferation, compared with nTg mice given DT. These experiments were performed two to five times with five mice per group per time point.

response to Ag trapped in the lung vasculature. For this purpose, $Ly6C^{hi}$ monocytes were sorted from the bone marrow (based on expression of CD11b and Ly6C and lack of expression of CD31) and adoptively transferred i.v. in CD11c DTR Tg mice given DT around the time of bead injection. As seen in **Fig. 3 F**, restoration of OVA-specific CD4+ T cell divisions could be accomplished by injection of bone marrow–purified $Ly6C^{hi}$ monocytes. Monocytes have also been shown to be precursors of $Langerin^+$ DCs in the lungs (Jakubzick et al., 2008) and Langerhans cells of the skin (Ginhoux et al., 2006), and DT treatment of CD11c-DTR might target these $langerin^+$ DCs directly, as they express high levels of CD11c. Therefore, we also addressed whether lung $langerin^+$ DCs were involved in this response by treating C57BL/6 langerin-DTR mice systemically with DT. In these mice, depletion of $langerin^+$ cells had no effect whatsoever on divisions of OVA-specific OTII T cells in the MLNs, which is in sharp contrast to the same treatment in CD11cDTR mice (**Fig. 3 G**). Together, these data therefore suggest that venous embolic Ags are filtered in the lung vascular bed and presented by monocyte-derived $CD11c^+$ DCs in the MLNs.

Induction of maturation of monocyte-derived DCs by microbial stimuli and trimeric CD40L increases effector potential of T cells without affecting T cell division

In all our experiments, we observed that inflammatory lesions around the OBs eventually resolved by day 8 of the response. This could be a result of the fact that OBs are seen as relatively harmless Ags, leading to a failure of functional maturation in monocyte-derived $CD11c^+$ DCs. To address this point, mice were injected with OBs that were also coated or not with LPS and/or trimeric CD40L or as a control with UBs. Trimeric CD40L is an effective agonist of CD40 on DCs and is known to induce DC maturation, particularly when a concomitant TLR agonist such as endotoxin is administered (Schulz et al., 2000). In mice receiving OB coated in combination with trimeric CD40L and LPS, there was a strong increase in the size of inflammatory lesions around the OBs (**Fig. 4 A**, inflammatory lesions at day 4 after bead injection). LPS co-coating of OB by itself was insufficient to obtain this effect (**Fig. 4, A and B**). This enhancing effect was maintained until day 16 after injection of beads, although inflammatory lesions became much smaller compared with those seen on day 4 (**Fig. 4 B**). Despite the occurrence of larger inflammatory lesions around OBs with LPS and sCD40L, at day 16 all inflammatory lesions were again resolved. Provision of these DC-maturing stimuli did not

affect the strength of T cell divisions in the MLN at day 4 after OB injection (**Fig. 4 C**). When the amount of cytokine production was measured in cultures of MLN, restimulated for 4 d in the presence of OVA Ag, the addition of LPS and/or sCD40L clearly enhanced the production of IFN-γ, whereas IL-4 remained at the detection limit of the ELISA (**Fig. 4 D**). To address whether CD40 and TLR4 triggering had to occur physically on the same particulate OB, mice received beads coated simultaneously with OVA, LPS, and anti-CD40 or mice received beads coated separately with OVA, LPS, or anti-CD40. OB and LPS-aCD40 OB-injected mice also received 2/3 UB to equalize the amount of beads and the amount of OVA Ag given between groups. As shown in **Fig. 4 E**, restimulated MLN cells secreted more IFN-γ when the beads were coated with OVA, LPS, and anti-CD40 simultaneously, compared with mice given a mixture of separately labeled OB, anti-CD40 beads, and LPS beads. We also addressed whether injection of OBs in conjunction with CD40 and TLR4 triggering led to induction of CTL responses to OVA-pulsed target cells. For this, we injected an equal amount of OVA-pulsed or unpulsed BM-DC targets, stained, respectively, with CMTMR and CFSE (Ritchie et al., 2000). As shown in **Fig. 4 F**, simultaneous triggering of CD40 and TLR4 on beads led to induction of OVA-specific lytic effectors that killed 50% of injected OVA-pulsed DCs, whereas injection of UBs or OVA-LPS–coated beads did not have this effect. Finally we also addressed the induction of humoral OVA-specific immunity by injection of these beads. We observed induction of OVA-specific IgG_1 after injection of OBs already when only TLR4 was triggered, whereas injection of UBs did not induce OVA-specific antibodies.

Discussion

In this paper, using a system of traceable Ag-reactive T cells specific to OVA, we report that the meshwork of small lung vessels allows the effective filtering of large embolic Ags followed by uptake of the Ag by monocyte-derived $CD11c^+$ DCs and Ag presentation to $CD4^+$ and $CD8^+$ T cells in the MLNs. Primed T cells subsequently return to the lung and form short-lived inflammatory lesions that are ultimately cleared. This response clearly depends on $Ly6C^+$ monocytes and $CD11c^{hi}$ DCs, as it is eliminated in mice with a conditional deletion of $CD11c^+$ cells and depleted of Gr1 $(Ly6C/G)^+$ cells, and restored by adoptive transfer of $Ly6C^{hi}$ monocytes (Jung et al., 2002). These data, therefore, identify a third filtering network for bloodborne Ags, in addition to the spleen and liver, that is specialized for immune induction against large embolic material. Strikingly, early after injection of embolic material, there was an accumulation $CD11c^+MHCII^+$ DCs surrounding the injected beads of which the origin can be debated. One possibility is that the resident interstitial DCs of the lung that can be found in alveolar septa would migrate to the occluded vessels and sample the vessel content. Numerous studies in mouse and rat have indeed demonstrated that alveolar septa contain DCs in close proximity to small diameter arterioles and alveolar capillaries (Sertl et al., 1986; Holt et al., 1988, 1992). However, these interstitial DCs have classically been described as sessile cells that fail to migrate to draining LNs and would merely restimulate already primed T cells during pulmonary delayed-type hypersensitivity–like reactions (Kradin et al., 1993; von Garnier et al., 2005). Another possible explanation would be that DCs accumulated on the injected beads from within the vessel lumen. Pulmonary vessels already contain a large marginating pool of DC precursors, possibly hardwired for Ag recognition at this site. This has been suggested by Suda et al. (1998), who demonstrated that the blood cannulated from the lungs of rats

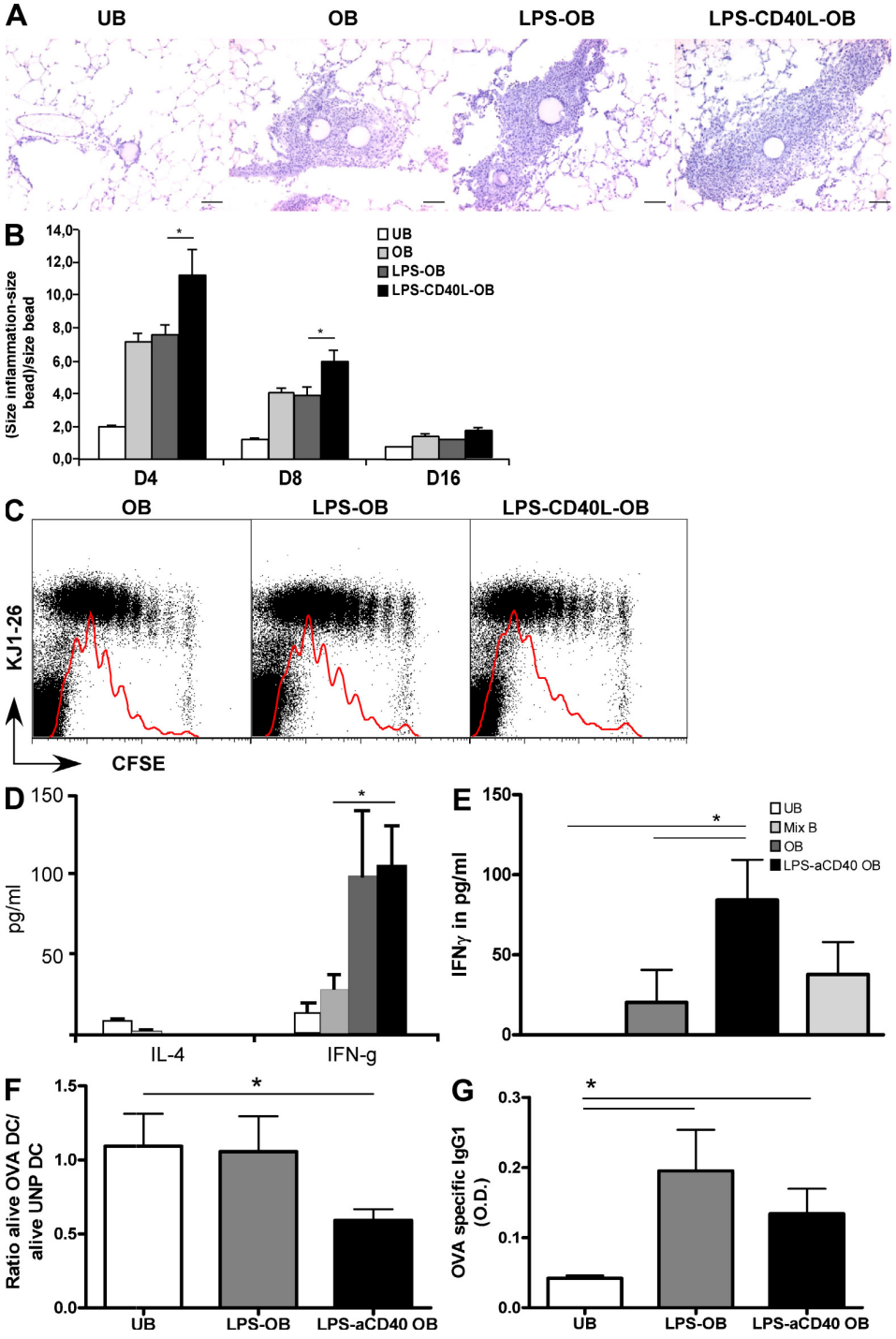

Figure 4. Effect of triggering TLR4 receptor and CD40 on Ag presentation to Ag trapped in the lung vasculature. (**A**) Mice received OVA-specific $CD4^+$ DO11.10 T cells at day -2, and UBs, OBs, OB co-coated with LPS (LPS-OB), or OB co-coated with LPS and trimeric CD40L (LPS-CD40L-OB) at day 0. 4 d later, inflammation surrounding the beads is shown in these groups. Bars, 100 µm. (**B**) Size of inflammation surrounding the beads in the lungs in these groups is shown at days 4, 8, and 16. Data are shown as mean (n = 4 mice/group) ± SEM. *, $P < 0.05$. (**C**) Representative flow cytometry analyses of (n = 4 per group) OVA-specific $CD4^+$ T cell divisions in draining lung MLNs at day 4 are shown. (**D**) Cytokine production in cultures of MLN, restimulated for 4 d in the presence of OVA Ag. The level of IL-4 was at the detection limit of the IL-4 ELISA (15 pg/ml). *, $P < 0.05$. Error bars represent statistical significance between these groups. (**E**) IFN-γ production by T cells was also analyzed in supernatant of restimulated MLN cells, from mice

injected with 3×10^3 UB, 10^3 OB combined with 2×10^3 UB, 10^3 LPS-aCD40 OB combined with 2×10^3 UB, or mixed beads. The mice receiving mixed beads were injected with 10^3 OB, 10^3 LPS-B, and 10^3 anti-CD40-B. Therefore, all mice received the same number of beads and the same amount of Ag. *, $P < 0.05$. Error bars represent statistical significance between these groups. (**F**) To analyze the OVA-specific effector T cell function in this model, mice were injected with UB, OB, or LPS- anti-CD40-OB and, after 6 d, injected subcutaneously with OVA peptide–pulsed DCs mixed with unpulsed DCs, respectively CMTMR and CFSE labeled. This graph shows the ratio of alive OVA peptide DCs versus unpulsed DCs, 4 d after injection of target DCs. *, $P < 0.05$. Error bars represent statistical significance between these groups. (**G**) In serum of these mice, OVA-specific IgG_1 levels were measured by ELISA. *, $P < 0.05$. Error bars represent statistical significance between these groups. These experiments were done three times with five mice per group.

contained much more DC precursors than vena cava–cannulated blood, and these cells obtained DC potential after culture in appropriate cytokines. Whether this sequence of DC differentiation from vascular precursors would occur in our system remains to be shown. Although all of these scenarios are not mutually exclusive, we favor a third potential source for bead-associated $CD11c^+$ DCs. Ag presentation in our system of i.v. embolization occurred as the result of Ag recognition by $Gr1^+$ (Ly6C/G) $CD11c^+$ cells that accumulated as early as 6 h after bead injection. These cells most likely represented monocyte-derived DCs and not pDCs, as they lacked expression of B220 and 120G8. The evidence for this comes from the co-expression of the fractalkine (CX3CR) receptor, Ly6C, and CD11c on these cells and the fact that T cell proliferation and development of inflammatory lesions in the lung were significantly reduced when mice were depleted of Gr1 $(Ly6G/C)^+$ cells but not by depletion of $120G8^+$ cells. In previous studies, $Ly6C^+$ monocytes have been shown to be precursors for inflammatory-type DCs, thus acquiring APC function and expression of the CD11c marker, and most likely represent the immediate precursor to nature's adjuvant, the immunogenic DC (Serbina et al., 2003; Le Borgne et al., 2006; Naik et al., 2006; León et al., 2007; Shortman and Naik, 2007; Kool et al., 2008). In the mouse, circulating monocytes can be discriminated into $Ly6C^+$ $CX3CR^{int}$ $CD11b^+$ inflammatory monocytes and $Ly6C^+$ $CX3CR^{hi}$ $CD11b^+$ monocytes (Geissmann et al., 2003). Several groups have now shown that both types of monocytes are also immediate circulating precursors for lung DCs but not steady-state lymphoid tissue DCs (Geissmann et al., 2003; Landsman et al., 2007; Varol et al., 2007; Jakubzick et al., 2008). Strikingly, $Ly6C^{hi}$ monocytes express the CCR2 receptor for MCP-1. Although we have not measured the level of this chemokine, others using an injection model of Schistosomal egg antigen or *Mycobacterium* purified protein derivate (PPD)–coated Sepharose beads could measure an early production of this chemokine by lung structural cells (Hogaboam et al., 1999). Supporting a crucial role for inflammatory monocytes in early granuloma formation, CCR2-deficient mice had a defect in early (day 1–2) granuloma formation. It will be interesting to study whether these CCR2-deficient mice also have a reduced DC accumulation around beads and delayed Ag presentation in our model. This could be a possibility, as CCR2 was shown to be crucial for DC recruitment to the other lung compartment, the airway mucosa, under inflammatory conditions (Robays et al., 2007). Alternatively, a population of $CX3CR^{med}$ $Ly6C^-$ monocytes has been shown to patrol the inside of the vessel wall (Auffray et al., 2007; Geissmann et al., 2008) We do not believe, however, that this subset was involved in presenting trapped embolic Ag in the lung, as the majority of $CX3CR^{gfp+}$ cells were $Ly6C^+$ and depletion of $Gr1^+$ cells abolished most Ag presentation, whereas adoptive transfer of $Ly6C^{hi}$ monocytes restored it. Systemic DT treatment of CD11c-DTR at the time of bead injection led to a reduction of $CX3CR1^+$

$Ly6C^+CD11c^+$ DCs and inflammatory $CX3CR1^+Ly6C^+CD11c^-$ monocytes around injected OB. These findings were very striking, as injected DT only kills cells expressing high level CD11c and circulating monocytes with this phenotype have not been described to be depleted in these mice (Geissmann et al., 2003; Landsman et al., 2007; Sapoznikov and Jung, 2008). The fact that DT has a very short half-life also did not support a model in which CD11c (and consequent DTR expression) was up-regulated on monocytes after recruitment to the beads, rendering monocytes sensitive to DT. We favor a model in which resident $CD11c^+$ DCs act as gatekeepers that chemo-attract the inflammatory DCs, which, in their turn, are the true vehicles of immunity. In support of this, we found that $CD11c^+$ DCs were a predominant source of MCP-1, the major chemokine attracting $CCR2^+$ inflammatory monocytes. In CD11c-DTR Tg mice, treatment with DT largely eliminated the presence of MCP-1 around the injected beads, explaining how DT treatment can also lead to a reduction in monocytes not expressing CD11c. Strikingly, a lot of the MCP-1 was found not associated with the DAPI nuclear signal. Previously, MCP-1 was found indeed to be able to mediate its chemotactic effects at a distance from its site of production, displayed on extracellular matrix proteoglycans (Palframan et al., 2001). However, the extracellular MCP-1 signal was completely eliminated in DC-depleted mice, suggesting that a $CD11c^{hi}$ cell was the predominant source for extracellular MCP-1. The exact subtype of lung DCs expressing MCP-1 in our model remains to be established. Resident $CD11c^+CD11b^+CD103^{neg}$ DCs are found in the lung interstitium and have been shown to be a prominent source of chemokines (Beaty et al., 2007). Recently also, Choi et al. (2009) identified a population of vascular $CD11c^+CD11b^+$ cells that probes the large systemic vessels. One surprising observation of this and other models of embolization, such as injection of *Schistosoma mansoni* soluble Ag-coated or *Mycobacterium* PPD Ag-coated Sepharose beads, was that inflammatory lesions around injected embolic material were very short lived (Chensue et al., 1994; Iyonaga et al., 2002). It is also known that parasites or parasite eggs that gain access to the venous system as part of their replicative cycle end up in the lung arterioles and capillaries, leading to formation of lung granulomas or transient pulmonary inflammatory infiltrates (called Löffler's syndrome). One explanation of these findings would be that, like in our model, monocyte-derived $CD11c^+$ DCs would not get the proper activation status to induce full blown effector cells, thus leading to a program of deletional proliferation of T cells. In support of this, IFN-γ cytokine production of dividing T cells of mice receiving OBs was not above the level seen in mice receiving UBs. Arguing against this possibility is the fact that injection of CD40 and LPS, two known activators of the DC system and its potential to produce IL-12p70 (Schulz et al., 2000), enhanced the size of the inflammatory lesions, production of IFN-γ by dividing T cells, production of IFN-γ by tissue-infiltrating T cells, and lytic activity of CD8 CTLs, but eventually these were still cleared from the lung. Also, models in which PPD-coated beads or S. mansoni eggs are injected would lead to full DC activation, but still these granulomata are cleared eventually (Iyonaga et al., 2002). Such a system to clear the lung interstitial tissue of overt inflammatory lesions makes evolutionary sense as this is the site of vital gas exchange (Lambrecht, 2006). Several mechanisms in the lung, such as the suppressive function of the nearby alveolar macrophage with its secretion of anti-inflammatory factors, such as TGF-β, IL-1RA, IL-10, and prostaglandins, might keep inflammation in check by down-regulating the Ag-presenting capacity of DCs (Holt et al., 1988; Iyonaga et al., 2002). Alternatively, early fibrosis occurring around injected beads could effectively shield off the Ag

so that it is further ignored by the immune system, eventually leading to apoptosis of T cells (Hogaboam et al., 1999). In conclusion, we have provided evidence for a highly active Ag sampling mechanism for embolic material trapped in the small vessels of the lung that can potentially lead to the generation of effector T cell responses when properly triggered. It is likely that this system developed during evolution to allow the immune system to effectively recognize large particulate Ags acquiring access to the venous blood by invasion, such as parasite eggs or worms, or after direct (traumatic) access to the bloodstream. When such Ags would be retained in the lungs without a possibility for Ag recognition, this could lead to a very effective way of pathogen immune subversion, as the spleen or liver, two other major sites of immune induction against blood particulate Ags, cannot access these large Ags and the pathogen could mutate during residence in the lung. It will be interesting to study whether certain pathogens take advantage of this system by blocking DC migration to the mediastinal nodes. It could also be that allogenic cells disrupted as a group of cells from freshly transplanted vascularized organs are presented via this route, as these vascularized grafts lack a direct connection with afferent lymph and the lung vascular bed is the first small vessel filter encountered. Clearly, this pathway of immune induction in the lung vasculature deserves further study.

Materials and methods

Mice. 6–8-wk-old female BALB/c (H-2d) and C57BL/6 mice were purchased from Harlan. OVA-TCR Tg mice (DO11.10) on a BALB/c background, OT-II and OT-I OVA-TCR Tg mice on a C57BL/6 background, CD11c-DTR Tg mice on a BALB/c background, and Langerin DTR Tg mice on a C57bl/6 background (Bennett et al., 2005) were bred at Erasmus University (Rotterdam, Netherlands) and University of Ghent (Ghent, Belgium). CX3CR-GFP on a C57BL/6 background was a gift from S. Jung (Weizmann Institute of Science, Rehovot, Israel; Jung et al., 2000). Mice were housed under specific pathogen-free conditions at the animal care facility at Erasmus University. All of the experimental procedures used in this study were approved by the Erasmus University Committee of Animal Experiments and the Animal Ethics Committee of the University of Ghent.

Reagents and antibodies. $OVA_{323-339}$ peptide was obtained from Ansynth Service B.V. OVA protein was obtained from Worthington Biochemical Corporation. CNBr-activated Sepharose 4B beads were obtained from Sigma-Aldrich. CFSE and CellTracker Orange (CMTMR) were obtained from Invitrogen. FITC-labeled anti-Gr1 (RB6-8C5) and PE-labeled KJ1-26 (clonotypic OVA-TCR) were obtained from Invitrogen, PE-Labeled anti-B220 (RA3-6B2), CD8α (Ly-2), Vb 5.1/5.2, and APC-labeled anti-CD4 (RM4-5) were purchased from BD. Unconjugated anti-CX3CR1 and goat anti–rabbit FITC were obtained from Acris Antibodies.

Intravascular injection of emboli. BALB/c or C57BL/6 mice received i.v. in the lateral tail vein 10^4 OVA-coated (OB) or noncoated (glycine; UB) CNBr-activated Sepharose 4B beads on day 0. Beads between 40 and 150 μm in diameter were prepared and coated with OVA, as described by others (Chensue et al., 1994), and were concentrated on a 40-μm cell strainer to remove smaller beads. Because of low OVA-TCR–specific naive T cell frequency in these wild type BALB/c mice, 10×10^6 live CFSE-labeled OVA-TCR Tg naive DO11.10 T cells were given i.v. 2 d before injection of the beads (day –2). OVA-specific TCR Tg T cells were collected from the lymphoid organs of naive 4–6-wk-old DO11.10, OT-I, or OT-II mice and stained with CFSE (Invitrogen), as previously described (de Heer et al., 2004). In some experiments, to address the role of DC maturation, mice also received OB co-coated with LPS (LPS-OB; LPS obtained from Sigma-Aldrich) or OB co-coated with LPS and trimeric CD40L (2 μg/ml sCD40L, 100 ng/ml LPS; LPS-CD40L-OB). Agonistic anti-CD40 was used at 200 ng/ml. Trimeric CD40L was a gift from Amgen.

Detection of the primary T cell response to OB injection i.v. On days 1, 2, 4, 8, and 16, MLNs, PLNs (axillary), spleens, and lungs were removed, and individual cell suspensions were prepared as previously described (Lambrecht et al., 2000). Cell suspensions were stained either with antibodies to CD4, CD8, KJ1-26 (specific anti-TCR antibody for the TCR recognizing OVA peptide in $I\text{-}A^d$), or Vβ5.1/5.2. Propidium iodide (Sigma-Aldrich) was added for exclusion of dead cells before analysis of CFSE profiles on a FACSCalibur flow cytometer using CellQuest (BD) and FlowJo software (Tree Star, Inc.). For measurement of cytokine responses, cell suspensions obtained from MLNs were cultured at 200,000 cells per well in the presence of 10 μg/ml OVA protein. IL-4 and IFN-γ were measured at day 4 by ELISA (BD).

Depletion of DCs and monocytes. In experiments to address the functional role of DCs in inflammation formation around beads, $CD11c^+$ cells were depleted by injecting 100 ng DT i.p. in CD11c-DTR Tg mice (van Rijt et al., 2005). The same dose is used in Langerin DTR mice to deplete the $Langerin^+$ cells. In experiments to address the functional role of pDCs, pDCs were depleted by 120G8 antibody injection (200 µg at the day of bead injection; provided by C. Asselin-Paturel, Shering-Plough, Dardilly, France; Asselin-Paturel et al., 2003). For monocyte depletion, anti-Gr1 i.p. injection was used, which was given at 200 µg on the same day of bead injection. In experiments to address if monocytes could restore immune responses in the DT-treated CD11c-DTR Tg mice, 3.5×10^5 $CD11b^+Ly\text{-}6C^+CD31^-$ monocytes sorted from bone marrow of RAG2 $\gamma c^{-/-}$ BALB/c mice (Geissmann et al., 2003) were injected i.v. on day 0 after OB or UB injection. To investigate whether DCs migrated to the draining LNs and present OVA, DCs were sorted from the MLN and lung from mice that were injected 4 d earlier with OB and control mice that were injected with UB. Macrophages were sorted from the lungs of these mice as a control of APCs. DCs and macrophages were subsequently brought in and co-cultured for 4 d with CFSE-labeled OT-I and OT-II cells to analyze their presenting capacity. Monocytes, DCs, and macrophages were sorted on a cell sorter (FACS-ARIAII; BD).

CTL response. C57BL/6 mice were injected with 10×10^6 unlabeled OTI T cells and, 2 d later, with UB, LPS-OB, or LPS-aCD40-OB. DCs were cultured for 6 d from bone marrow of C57BL/6 mice with GM-CSF. At day 6, half of the DCs were pulsed with OVA peptide (10 µM SIINFEKL) for 2 h. These cells were harvested and labeled with 10 µM CMTMR (10×10^6 cells/ml) according to the manufacturer's protocol (Invitrogen). The unpulsed DCs were harvested and fluorescently labeled with 10 µM CFSE. Both types of DCs were mixed in a 1:1 ratio and injected 6 d after the subcutaneous bead injection. After another 4 d, the inguinal LNs were dissected and the migrated $CMTMR^+$ DCs and $CFSE^+$ DCs were analyzed by FACS. Serum was collected from these mice and OVA-specific IgG_1 levels were detected with ELISA (BD).

Confocal microscopy. Confocal analysis was performed on 6-µm cryostat sections of lungs stained with anti-CX3CR1/goat anti–rabbit FITC (Acris Antibodies), anti-Gr1 FITC, anti-B220 PE, Ly-6C biotin-streptavidin Alexa Fluor 555, CD11c Alexa Fluor 647, rabbit anti-MCP-1 (Abcam)/donkey anti–rabbit Cy3 (Jackson ImmunoResearch Laboratories, Inc.), and 120G8 coated with Quantum dots (Micro Probe, Inc.). Sections were collected on a confocal laser microscope (LSM-710; Carl Zeiss, Inc.) and analyzed using Imaris 5.0 software (Bitplane).

Immunohistochemistry and analysis of inflammation surrounding beads. For immunehisto-chemistry analysis of the inflammation, 6-µm cryostat sections of lungs were stained with CD11c, CD11b, and MHCII. For inflammation size, hematoxylin-counterstained lungs were analyzed with an image analysis system (Quantimed; Leica), whereby 30 beads per mice were measured, dividing the surface size of the bead (in micrometers squared) by the surface size of inflammation and bead together (in micrometers squared). 30 beads were chosen to attain a mean bead diameter, as slides were 6 µm. Online supplemental material. Fig. S1 shows T cell divisions in spleen and draining LNs of the lung, liver, and periphery at days 2 and 4 after bead injection. Fig. S2 depicts a confocal image of a section of a lung 24 h after bead injection, stained with CD11c, GR1, and B220. Fig. S3 shows in vitro T cell proliferations of OT-I cells co-cultured with sorted DCs and macrophages from MLN and spleen 24 h after beads injection. In Fig. S4, the analysis of the size of the infiltrates after GR1 depletion, as well as the T cell proliferations after pDC depletion at day 4 after bead injection, is depicted.

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Recent findings

Current reports on antigen presentation of blood-derived antigens confirm our results on the importance of inflammatory DCs. We used markers such as Ly6C and CX3CR1 to show that the antigen presenting cells were derived from monocytes and could be therefore considered as inflammatory DCs. Nowadays, markers such as MAR-1 and CD64 are used to discriminate between conventional DCs or recruited mo-DCs. A recent paper using Ly6C demonstrated that,

in the spleen, monocytes can present antigen with or without upregulation of CD11c (Drutman et al., 2012). We also observe $CD11c^{low/neg}$ monocytes surrounding the embolic antigen in the lung (figure 3B), but we did not investigate if CD11c was expressed/ upregulated on monocytes presenting the antigen in the MLN. The inflammatory areas shown in this chapter consist mainly of T cells and DCs. Regulatory T cells were also found in the infiltrates ($CD25^{+}$, $FoxP3^{+}$). Interestingly, depleting Tregs by administration of diphtheria toxin into DEREG-DTR mice, at the time of bead injection, lead to a reduction in the size of inflammatory areas around the beads (unpublished data). Various groups have reported the importance of Tregs in controlling the immune response in sarcoidosis. Although Taflin showed that Tregs proliferated and accumulated within the granulomas (Taflin et al., 2009), Tregs in sarcoidosis patients have an impaired *ex vivo* immunosuppressive function and survival than Tregs of healthy donors (Broos et al., 2012).

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Supplemental material

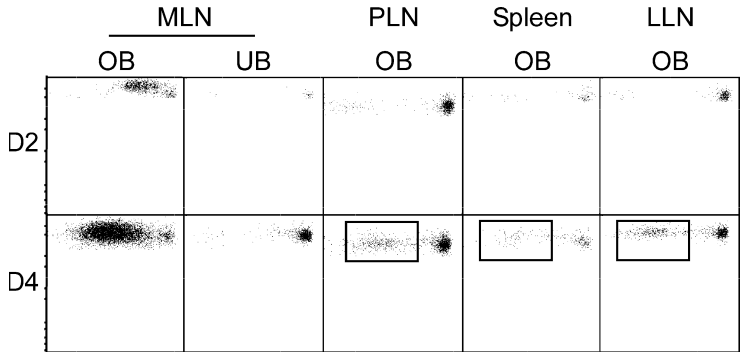


Figure S1. Mice were injected with CFSE-labeled OVA-specific T cells and, 2 d later, injected with either UB or OB i.v. At 2 and 4 d after bead injection, lung draining LNs (MLNs), PLNs, liver LNs (LLNs), and spleen were analyzed by FACS for T cell proliferation. This experiment was performed twice.

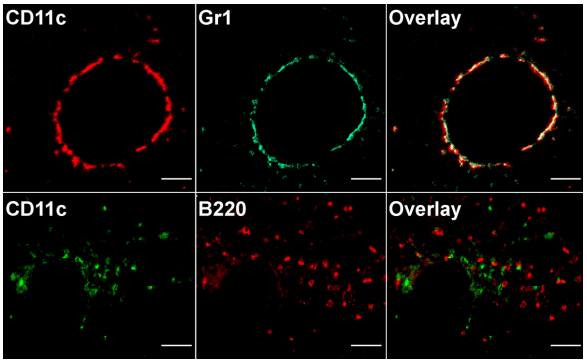


Figure S2. Confocal microscopy of frozen lung sections taken 24 h after injection of OBs in mice that received a cohort of DO11.10 T cells. (Top) Slides were double stained with anti-CD11c PE (red) and anti-Gr1 FITC (green). (Bottom) To discriminate pDCs, slides were also stained for $CD11c^{+}$ cells (FITC, green) in combination with anti-B220 PE (red). This experiment was performed twice. Bars, 10 µm.

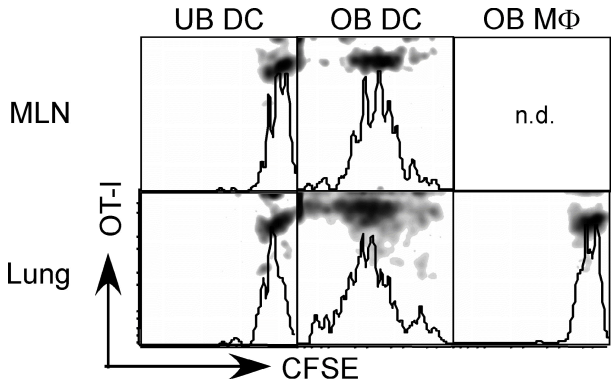


Figure S3. DCs and macrophages were sorted from MLN and lung from mice injected with UB or OB. These APCs were subsequently put in coculture with CFSE-labeled OT-I cells for 4 d. Autofluorescent alveolar macrophages were not detected (n.d.) in the MLNs. This experiment is performed twice.

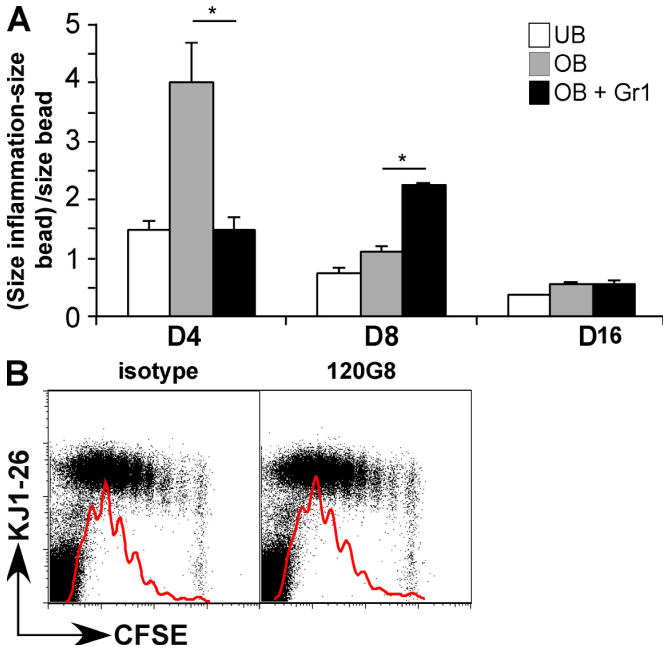
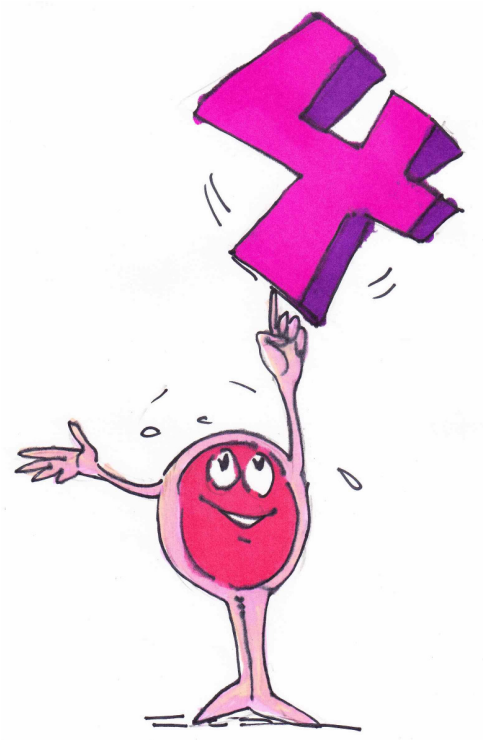


Figure S4. Depletion of monocytes and pDCs by using GR1 depleting antibody around the time of bead injection showed a reduction of inflammatory infiltrate around beads at day 4, whereas treatment with pDC-depleting antibody (120G8) did not reveal differences in antigen presentation. (**A**) Measurement of the size the inflammatory infiltrate at day 4, 8 and 16 around beads in the lungs of mice receiving either UBs (white bars), OBs plus isotype (OB, gray bars), or OBs plus anti-Gr-1 (OB Gr1, black bars). Data are shown as mean (n = 4 mice/group) ± SEM *, $P < 0.05$. (**B**) Effect of depletion of 120G8 positive pDCs. Comparison of CFSE labeled OVA specific $CD4^+$ DO11.10 division profile in draining MLNs, in mice treated with 120G8 (for pDC depletion) or isotype i.p. at day 0 and injected with OB, measured at day 4. Both experiments were performed twice.



Ursodeoxycholic acid suppresses eosinophilic airway inflammation by inhibiting the function of dendritic cells through the nuclear farnesoid X receptor.

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We investigated the immunomodulatory properties of ursodeoxycholic acid (UDCA) in allergic asthma, since UDCA is effective in reducing liver eosinophilia primary biliary cirrhosis. So far, no other groups have investigated the immunosuppressive properties of UDCA outside the gastro-intestinal tract. We show that UDCA alters DC/ T cell interaction, thereby reducing inflammation in the lungs.

Ursodeoxycholic acid suppresses eosinophilic airway inflammation by inhibiting the function of dendritic cells through the nuclear farnesoid X receptor

Abstract

Background: Ursodeoxycholic acid (UDCA) is the only known beneficial bile acid with immune-modulatory properties. Ursodeoxycholic acid prevents eosinophilic degranulation and reduces eosinophil counts in primary biliary cirrhosis. It is unknown whether UDCA would also modulate eosinophilic inflammation outside the gastrointestinal (GI) tract, such as eosinophilic airway inflammation seen in asthma. The working mechanism for its immunomodulatory effect is unknown.

Methods: The immunosuppressive features of UDCA were studied *in vivo*, in mice, in an ovalbumin (OVA)-driven eosinophilic airway inflammation model. To study the mechanism of action of UDCA, we analyzed the effect of UDCA on eosinophils, T cells, and dendritic cell (DCs). DC function was studied in greater detail, focussing on migration and T cell stimulatory strength *in vivo* and interaction with T cells *in vitro* as measured by time-lapse image analysis. Finally, we studied the capacity of UDCA to influence DC/T cell interaction.

Results: Ursodeoxycholic acid treatment of OVA-sensitized mice prior to OVA aerosol challenge significantly reduced eosinophilic airway inflammation compared with control animals. DCs expressed the farnesoid X receptor for UDCA. Ursodeoxycholic acid strongly promoted interleukin (IL)-12 production and enhanced the migration in DCs. The time of interaction between DCs and T cells was sharply reduced *in vitro* by UDCA treatment of the DCs resulting in a remarkable T cell cytokine production. Ursodeoxycholic acid-treated DCs have less capacity than saline-treated DCs to induce eosinophilic inflammation *in vivo* in Balb/c mice.

Conclusion: Ursodeoxycholic acid has the potency to suppress eosinophilic inflammation outside the GI tract. This potential comprises to alter critical function of DCs, in essence, the effect of UDCA on DCs through the modulation of the DC/T cell interaction.

Introduction

Bile acids are end products of cholesterol metabolism and have essential functions in controlling the secretion of bile fluid, regulating cholesterol elimination by the liver, and promoting absorption of lipids and fat-soluble vitamins, through detergent properties in the gut. Ursodeoxycholic acid (UDCA) is a naturally occurring dihydroxy bile acid that is only found in trace amounts (1–3% of bile acids) in human bile, but has strong choleretic activity and thus prevents cholestasis. Although UDCA is commonly used in the treatment for primary biliary cirrhosis (PBC) an autoimmune disease, the mechanisms behind this beneficial effect are not fully understood. In addition to promoting bile secretion, it has been suggested that UDCA has immunomodulatory properties (1–5), such as the suppression of immunoglobulin production; suppression of cytokine synthesis by lymphocytes; downregulation of MHC I expression on hepatocytes; inhibition of mast cell activation (6); inhibition of gall bladder inflammation (7, 8); and suppression of eosinophil activation and degranulation, typical of PBC (9, 10). The immunosuppressive effects of UDCA are, at least in part, mediated through the activation of

the glucocorticoid receptor (GR) and inhibition of NF-κB-dependent transcription through GR–p65 interaction (11–13). The purpose of this study was to observe whether UDCA also has immunomodulatory effects outside the gastrointestinal (GI) tract. To study this, we turned to the lung because this organ is also exposed to the outside world, lined with mucosal epithelial cells, and has been shown to develop eosinophilic inflammation when triggered with allergens. In this model, we report that intratracheal administration of UDCA strongly suppresses the development of airway inflammation induced by allergen challenge.

Results

Ursodeoxycholic acid treatment reduces eosinophilic airway inflammation and IL-13 production

Ursodeoxycholic acid was used as a topical therapeutic drug in a mouse model of acute allergic inflammation in which we administered 30 μg UDCA in diluent (=PBS) (1 mM 80 μl) or diluent approximately 30 min before each OVA aerosol challenge during 30 min in OVA-immunized mice. Twenty-four hours later, animals were killed, and UDCA-treated animals (OVA–alum/UDCA/OVA group) demonstrated a significant reduction in the percentage and the number of BAL eosinophils compared with the controls (OVA–alum/PBS/ OVA group) (**Fig. 1A,E**). Matching the reduction in eosinophils, we found that the number and percentage of DCs and T cells (**Fig. 1B,C,F,G**) were also lower in the UDCA-treated group compared with the control (OVA–alum/PBS/OVA) group. Next, we measured BHR. A significant less response to MCH was observed in the UDCA-treated animals (OVA–alum/UDCA/OVA group) as compared to PBS-treated animals (OVA–alum/PBS/OVA group). Phosphate-buffered saline–alum controls showed no signs of BHR (**Fig. 1I**). To address the effect of UDCA treatment on cytokine production, the mediastinal LNs draining the lungs were stimulated with OVA *in vitro*. After 4 days of OVA restimulation, levels of IL-13 (**Fig. 2B**) were markedly reduced, while other measured cytokines like IL-4 (**Fig. 2A**), IL-5 (**Fig. 2C**), and IL-10 (**Fig. 2D**) remained unchanged in OVA–alum/UDCA/OVA group compared with OVA–alum/PBS/OVA group. In line with the reduced eosinophilic BAL inflammation, significantly lower levels of IL-13 in the BAL were observed in OVA–alum/UDCA/OVA group (104 ± 3.7 pg/ml vs 140 ± 7.1 pg/ml, compared with OVA–alum/PBS/OVA group, $P = 0.006$). The controls, PBS–alum/UDCA/OVA group, and PBS–alum/PBS/OVA group had much lower IL-13 concentrations that fell in the same range as the OVA–alum/UDCA/OVA group (**Fig. 2E**). Next, we measured intracellular IFN-γ in BAL and MLN and observed a trend ($P = 0.06$) of increased numbers of IFN-γ-positive CD4 T cells in BAL and significantly ($P = 0.03$) more IFN-γ-positive CD4 T cells in MLN in the UDCA-treated animals (OVA–alum/UDCA/OVA group) when compared with PBS-treated animals (OVA–alum/PBS/ OVA group) (**Fig. 2F–G**).

Ursodeoxycholic acid induce IL-12 production in DCs and has no toxic effect on eosinophil, DC survival, and T cell cytokine production

A detailed in vitro characterization of UDCA-treated eosinophils, DCs, and T cells is shown in Fig. S1 with the conclusion that UDCA is not harmful for eosinophils and DCs (**Fig. S1A,B**) and does not influence T cell cytokine production (**Fig. S1C–E**). As we found that UDCA enhances migration of bone marrow- derived DCs after i.t. injection (**Fig. S1F**), yet at the same time suppresses the potential of these cells to prime for Th_2-dependent eosinophilic airway

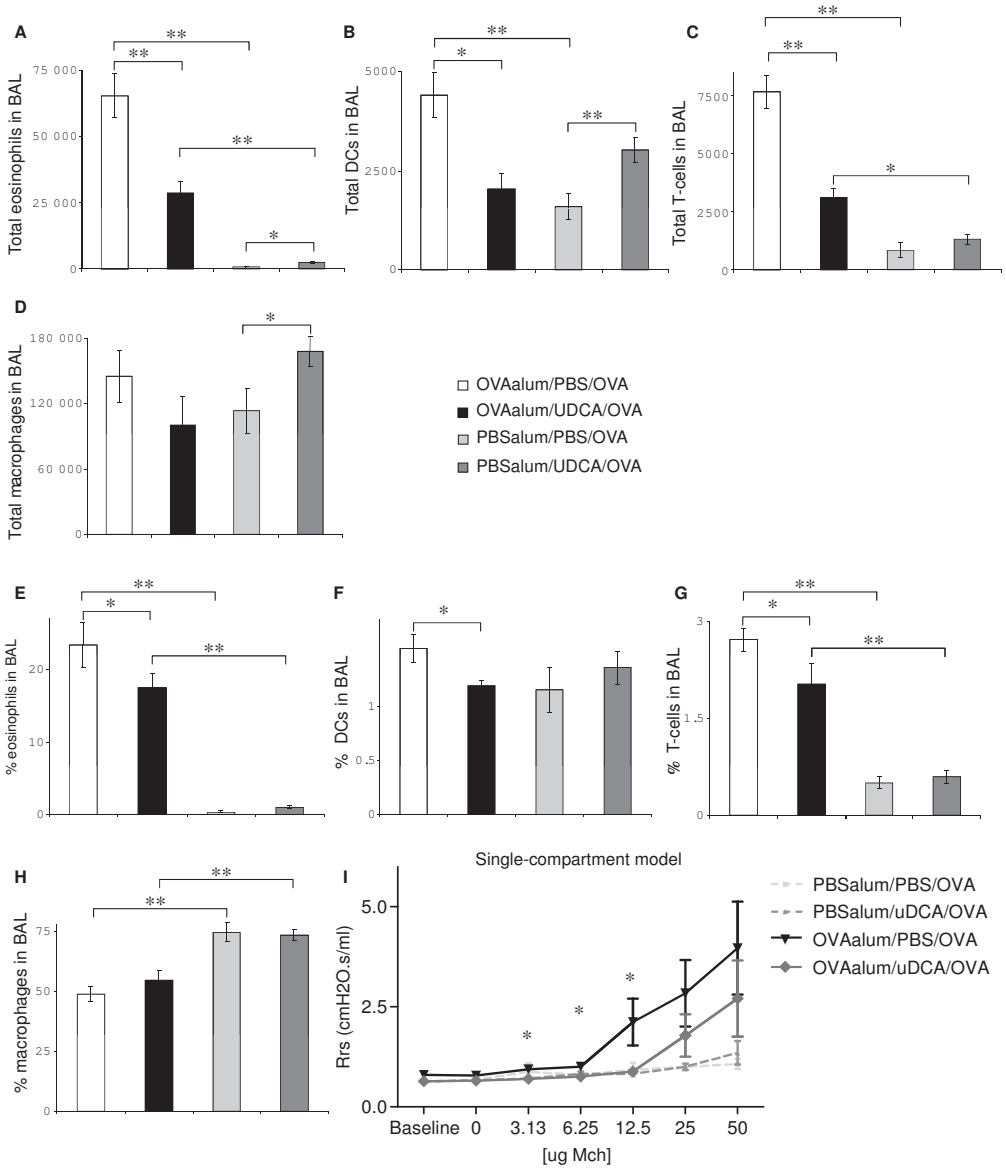

Figure 1 Impaired eosinophilic airway inflammation after ursodeoxycholic acid treatment. Model of allergic airway inflammation in ovalbumin-sensitized mice. Bronchoalveolar lavage cells were stained for eosinophils (CCR3-positive cells) and T cells (CD3-positive cells), as well as for B cells (data not shown) and macrophages and DCs. (**A** and **E**) eosinophils (cell numbers and percentage); (**B** and **F**) DCs (cell numbers and percentage); (**C** and **G**) T cells (cell numbers and percentage); (**D** and **H**) macrophages (cell numbers and percentage); (**P < 0.01 and *P < 0.05 n = 6 animals per group); (**I**) bronchial hyperreactivity measurements (*P < 0.05 n = 4–6 animals per group).

inflammation, we sought to further identify the mechanism by which Th_2 development was abolished. Previous studies in our group have identified that high-level production of IL-12 by GM-CSF-cultured DCs abolished their potential to induce Th_2 immunity, via promotion of a counterregulatory Th_1 response (18, 19). IL-12 is known to be a key inducer of cell-mediated immunity. In addition, IL-12 stimulates the differentiation of CD4-positive Th cells in IFN-γ-producing Th_1 cells (18). Incubation of DCs with increasing UDCA concentrations revealed a concentration-dependent increase in IL-12 production (**Fig. 3A**), promoting the induction of IFN-γ in responding T cells (**Fig. 3C**), while suppressing induction of Th_2 cytokine IL-13 (**Fig. 3B**), and supports the migration of DCs (**Fig. S1F**).

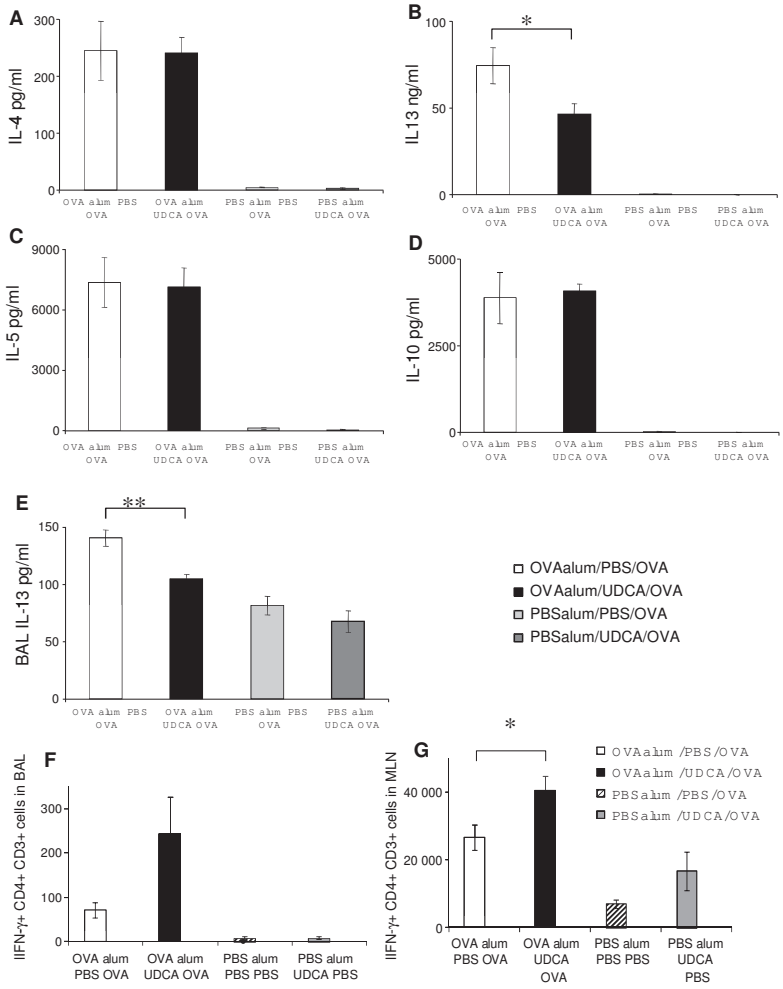

Figure 2 Reduced interleukin (IL)-13 production after ursodeoxycholic acid (UDCA) treatment. Mediastinal lymph nodes (LN) cell suspensions were restimulated *in vitro* for 4 days with ovalbumin and assayed for IL-4 (**A**), IL-13 (**B**), IL-5 (**C**), IL-10 (**D**) production using enzyme-linked immunosorbent assay. The level of the IL-13 promoting Th_2 cytokines was significantly reduced in mice treated with UDCA. Levels of other cytokines seem to be unchanged in the UDCA-treated group. IL-13 levels (**E**) in bronchoalveolar lavage (BAL) fluid were lower in UDCA-treated animals compared with the diluent-treated animals (**F**) and (**G**) intracellular interferon-γ in CD4 T cells in, respectively, BAL cells and MLN cells (**P < 0.01 and *P < 0.05).

Ursodeoxycholic acid-treated DCs were less effective in inducing asthma

We employed experiments demonstrating that intratracheal administration of UDCA reduces the number of DCs in allergen-challenged lungs. In previous studies, we identified lung DCs as key pro-inflammatory cells that are necessary and sufficient for Th_2 cell stimulation during ongoing airway inflammation (20). To test whether UDCA interferes with the potential of DCs to induce eosinophilic airway inflammation, we used an adoptive transfer model of DCs to immunize the animals, bypassing OVA–alum immunization (21). Myeloid DCs were grown for 8 days from the bone marrow in GM-CSF, were subsequently pulsed with OVA or OVA in the presence of UDCA or PBS, and then $1x10^6$ DCs were injected into the trachea of mice. After this treatment, mice were given three additional OVA aerosol challenges. As previously reported (22), the adoptive i.t. transfer of OVA-pulsed mDCs leads to Th_2 priming, and eosinophilic airway inflammation subsequently develops upon OVA aerosol challenge 10 days later. As

expected, in mice receiving unpulsed PBS DCs or UDCA DCs, only a few inflammatory cells were observed in the BALF (**Fig. 4A**). Mice immunized with OVA-pulsed DCs demonstrated a strong cellular recruitment of eosinophils and macrophages in the BALF (**Fig. 4A, B**). Ursodeoxycholic acid treatment of OVA-pulsed DCs abolished their potential to induce airway eosinophilia and macrophage influx into the lung. There was also a trend toward reduced T cell (**Fig. 4C**) and DC (**Fig. 4D**) influx, but this failed to reach statistical significance.

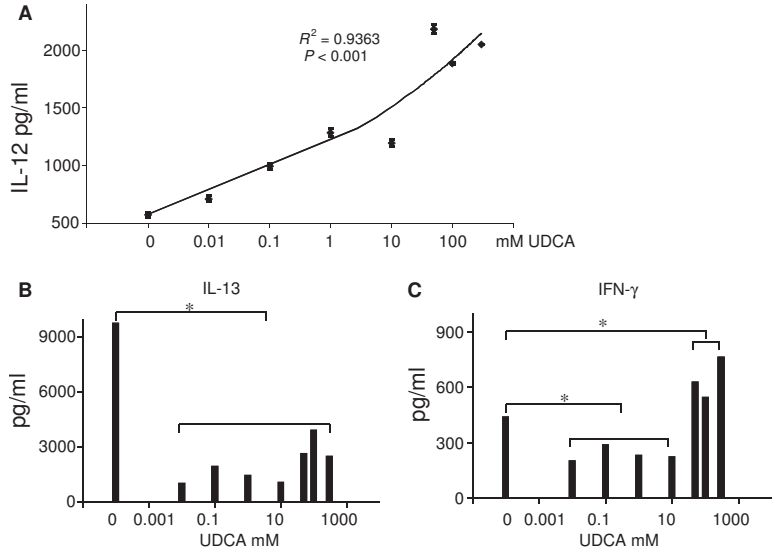

Figure 3 Ursodeoxycholic acid (UDCA) promotes the interleukin (IL)-12 production of DCs in a concentration-dependent manner (**A**). The effect of UDCA treatment on DCs was studied in the context of the interaction between T cells and DCs. Levels of T cell cytokines were reduced when UDCA-treated DCs were used. Reduced levels in the production of IL-13 (**B**) were shown for concentrations up to 300 μM UDCA. This concentration is not toxic for DCs. Low doses of up to 10 μM of UDCA-treated DCs reduced the production of T cell cytokine interferon (IFN)- γ (**C**). Ursodeoxycholic acid concentrations of 30 μM and more induce a stronger IFN-γ response (*P < 0.05).

Shorter interaction time between DCs and T cells when DCs were treated with UDCA

Ursodeoxycholic acid has been shown to induce higher levels of IL-12 production in an UDCA concentration–dependent way. The migration of bone marrow-derived DCs after i.t. injection was increased. These phenomena, and the immunosuppressive action of UDCA in an allergic airway model, suggest that more factors are involved in affecting the DC/T cell interaction. It has become possible to study the duration of interaction as a marker for synapse formation between DCs (C57bl/6) and antigen-specific T cells *in vitro* (17). Untouched naive CD4-positive T cells were isolated from LNs obtained from OTII (OVA-specific TCR transgenic) mice carrying the Vα2 TCR chain, were cultured with MHCII-positive DCs for 40 min and formed cellular interaction. Unpulsed DCs showed short interactions between DCs and T cells, while OVA protein (100 μg/ml)–pulsed DCs showed long interactions between DCs and T cells. This was used as a method control. A long interaction time was also seen when DCs were pulsed with preprocessed OVA peptide. However, significantly shorter interactions were observed when OVA peptide (1 μg/ml)–pulsed DCs were treated with UDCA (10 μM) compared with saline (**Fig. 5A**).

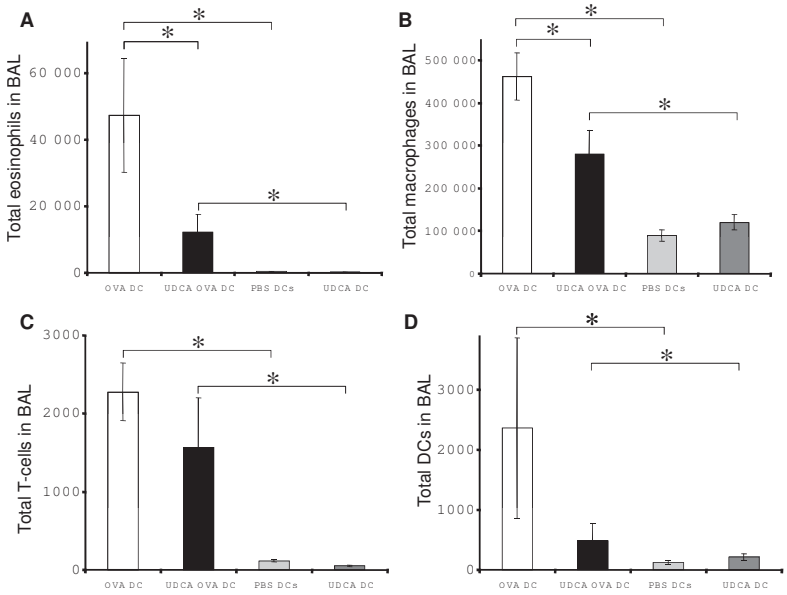

Figure 4 Ursodeoxycholic acid (UDCA)-treated dendritic cells (DCs) are less effective in inducing a Th2 response. Effects of *in vitro* UDCA treatment on the potential of mDCs to induce eosinophilic airway inflammation. On day 0, mice received an i.t. injection of diluents ovalbumin (OVA)-DCs, UDCA OVA-DCs, diluent DCs, or UDCA DCs. From days 10–13 onwards, mice were OVA aerosol– challenged. Total number of eosinophils in bronchoalveolar lavage (BAL) (**A**); total number of macrophages in BAL (**B**); total number of T cells (**C**); and total number of DCs (**D**). Data indicate mean ± SEM. *P < 0.05 n = 6 animals per group.

Dendritic cells express transcription factor farnesoid X receptor

Bile acids are ligands of the FXR, a nuclear receptor that mediates, among others, hepatic bile acid synthesis (23, 24). Fluorescence activated cell sorting (FACS) analysis indicated that the FXR is expressed on DCs grown in GM-CSF (**Fig. 5B**), suggesting the possibility that the suppressive effects of UDCA on DCs are partly mediated via FXR.

Ursodeoxycholic acid promotes DC-driven Th_1 development by acting on the farnesoid X receptor

We finally wanted to find the molecular mechanism by which UDCA promotes Th_1 induction and suppresses airway eosinophilia. To study whether FXR is involved in mediating the effects of UDCA on DC function, we grew myeloid DCs from FXR knockout mice and their wild-type littermates (C57bl/6). The DCs were stimulated with OVA (100 µg/ml) only, OVA and UDCA (10 µM), or PBS, and UDCA. Subsequently, $1x10^6$ DCs were injected intratracheally to sensitize C57bl/6 mice. Ten days later, the mice were challenged with OVA aerosols on three consecutive days. Twenty-four hours later, animals were killed, and UDCA-OVA-WT-DC-treated animals demonstrated no significant reduction in the number of BAL eosinophils, macrophages, and DCs compared with the DC-treated control animals. Moreover, UDCA-OVA-FXR KO-DC-treated animals demonstrated no significant differences in the number of BAL

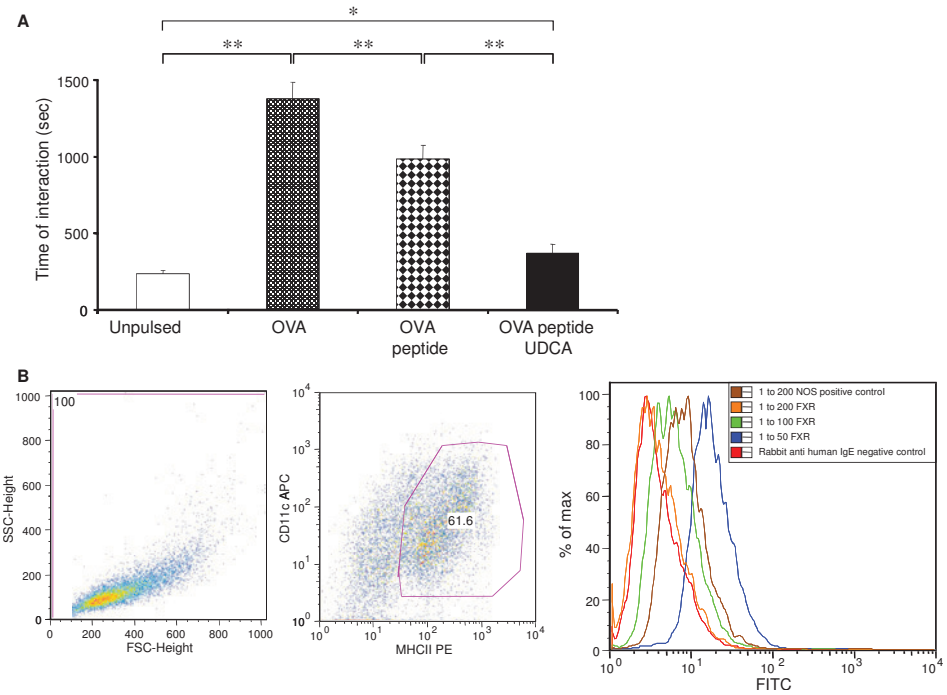

Figure 5 The duration of interaction between T cells and dendritic cells (DCs) is strongly reduced if DCs are treated with ursodeoxycholic acid (UDCA), and DCs express farnesoid X receptor (FXR). The duration of interaction between T cells and DCs is strongly reduced if DCs are treated with UDCA. (**A**). Bile acid acts as a ligand on the transcription factor FXR, a nuclear receptor for bile acid. GM-CSF-cultured bone marrow- derived DCs stained positive for FXR receptor, as indicated by FACS analyses with rabbit anti FXR antibodies. As a negative control, we used rabbit antihuman IgE, and as a positive control, we used NOS antibodies (**B**).

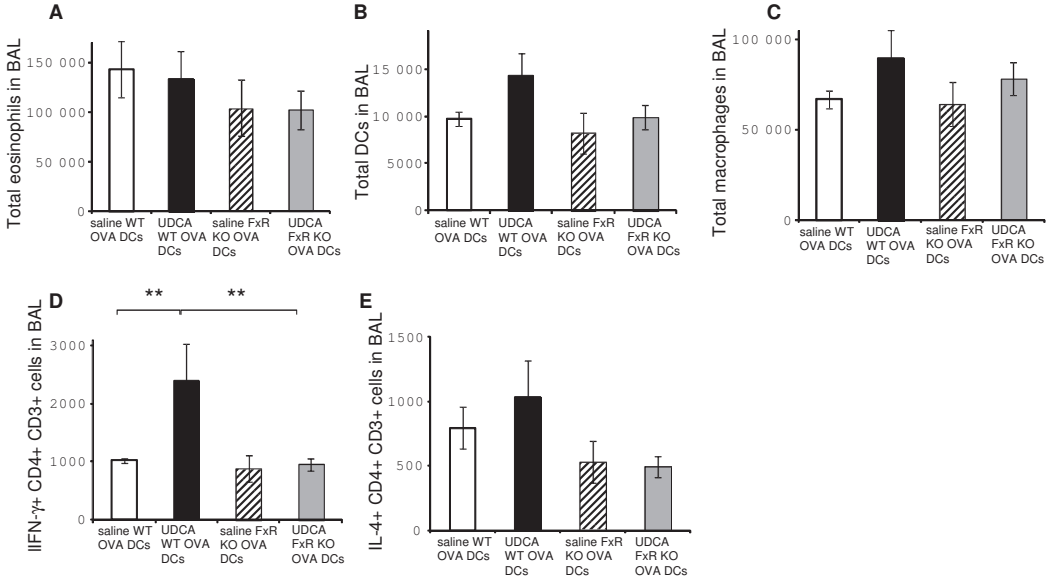

Figure 6 Higher interferon (IFN)-γ-positive T cell numbers in eosinophilic airway inflammation after ursodeoxycholic acid (UDCA) treatment. A model of asthma in ovalbumin (OVA)-sensitized mice. Bronchoalveolar lavage (BAL) cells were stained for eosinophils (CCR3-positive cells) and macrophages and DCs (**A**; eosinophils) (**B**; DCs) (**C**: macrophages). Bronchoalveolar lavage cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of Golgistop for 4 h. T cell cytokine production as measured by intracellular cytokine staining showed significantly ($P = 0.006$) higher IFN-γ -positive T-cell numbers in the UDCA-OVAWT- DC group compared with saline-OVA-WT-DC group and compared with saline-OVA-farnesoid X receptor-KO-DC group (**D**). No other differences were observed in the number of IFN-γ-positive T cells. Interleukin-4-positive T cells showed no differences between the groups (**E**) (**$P < 0.01$ n = 6 animals per group).

eosinophils, macrophages, and DCs compared with the saline-OVA-FXR-KO-DC-treated control animals (**Fig. 6B–D**). To check for Th polarization, BAL T cell cytokine production was estimated. Intracellular cytokine staining showed significantly (P = 0.006) higher IFN-γ-positive T cell numbers in the UDCA-OVA-WT-DC group compared with saline-OVA-WT-DC group and compared with saline-OVA-FXR KO-DC group (**Fig. 6E**). No differences were observed in the number of IFN-γ-positive T cells between saline-OVA-WT-DC group and saline-OVA-FXR-KO-DC group and between saline-OVA-FXR-KO-DC group and UDCA-OVA-FXR-KO-DC group. *In vivo*, there was no difference in the number of IL-4-positive T cells between the groups (**Fig. 6F**).

Discussion

Ursodeoxycholic acid modulates cellular and immunological processes *in vivo* and *in vitro*. Here, we have shown that UDCA treatment lowers the production of T cell cytokines following DC-driven activation *in vitro*. Moreover, UDCA treatment *in vivo* in the lung reduces tissue eosinophilia in mice in an allergic airway inflammation model. Ursodeoxycholic acid affects DCs and supports the production of IL-12 (Fig. 3A), suggesting that it is responsible for a pro-Th_1 T-cell cytokine induction, in a process requiring the FXR (25). Previous studies in our group have identified that high-level production of IL-12 by GM-CSF-cultured DCs abolished their potential to induce Th_2 immunity, via promotion of a counter-regulatory Th_1 response (18, 19). Higher numbers of intracellular IFN-γ-positive CD4 T cells in MLN suggest a change in favor of Th_1 balance because of the UDCA treatment. This is supported by *in vitro* data that DCs treated with UDCA are less capable to induce IL-13 (Th_2) response and that low concentrations of UDCA had similar effects on IFN-γ but higher concentrations induce and support IFN-γ. Intratracheal administration of UDCA could also act on structural cells of the airways that are responsible for the production of IL-25 and IL-33, both important for the recruitment of eosinophils (26) independent of T cells, and these cytokines are as well important for the recruitment of type 2 innate lymphoid cells (ILC2) (27) and may also act indirectly to suppress T-cell activation. However, in the *in vitro* experiments, we observed lower levels of Th_2 (IL-13) and increasing Th_1 (IFN-γ) cytokines, suggesting a direct immunosuppressive effect of UDCA on DC-driven activation of T cells. This is in line with previous observations (6). Somewhat surprisingly, there was a change in the balance of polarizing cytokine production in favor of IL-12, because the levels of IL-10 measured in DC supernatants were at the detection limit of the ELISA. The most likely explanation for the observed reduction in Th cytokine production was that the duration of the interaction between T cells and DCs was significantly reduced when DCs were treated with UDCA. This supports the idea that UDCA targets the DC. The outcome of an immune response after T cell/DC interaction is dependent on its duration, Ag levels, and T-cell numbers as recently shown using drugs that block the interaction between DCs and T cells (28). The effect of UDCA on suppressing allergic eosinophilic inflammation could be very well explained by the effects on DCs. Allergic airway inflammation is highly DC dependent, DCs playing an important role in the induction and maintenance of allergic airway inflammation (22, 29, 30). Another good target candidate for UDCA could be the eosinophils. In humans, there is strong evidence that UDCA prevents the degranulation of eosinophils in patients with PBC after 4 weeks of UDCA (10). Evidence from mouse eosinophils suggests that they do not degranulate in OVA-driven allergic airway inflammation models (31). We failed to see an effect of UDCA on eosinophil degranulation (data not shown). The effect of UDCA on DCs required

the nuclear FXR. This receptor has been described in peripheral blood mononuclear cells (PBMCs), including T cells and monocytes at the protein level and mRNA level, and it is inhibited by pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1 release during the acute phase response (32). Farnesoid X receptor is known as a receptor for bile acid metabolism, and our finding of FXR expression on bone marrow-derived DCs strongly supports the idea that DCs are targets for UDCA. Ursodeoxycholic acid activates the FXR and may be either an agonist or an antagonist (23, 24). To prove the functionality of FXR, we used FXR-deficient bmDCs or WT bmDCs and showed that sensitization with either FXR-KO bmDCs or WT bmDCs has the capacity to induce eosinophilic inflammation in C57bl/6 animals. However, the UDCA-induced promotion of Th_1 responses by DCs required the FXR. We failed, however, to observe a direct immunosuppressive effect of UDCA on eosinophil counts in WT DCs in C57bl/6 mice. This could be because of strain differences between C57bl/6 and Balb/c (33). Other ligands of the FXR are chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA). However, we found that these compounds were cytotoxic for DCs and for T cells (our unpublished observations). This cytotoxic effect was already reached at concentrations of 10 and 50 μM (data not shown). Our present results are another example of an unexpected orphan nuclear receptor–ligand interaction that has the capacity to suppress allergic inflammation. Other possible receptors are the PPAR-gamma receptor (34), the vitamin D receptor, and the aryl hydrocarbon receptor (35). As these nuclear receptors often form heterodimers with each other and other nuclear transcription factors, it will be very interesting to see how the FXR is regulated in DCs. Although UDCA has been shown to reduce eosinophilic functions in humans with PBC, our data suggest that UDCA might also affect DC function in the gut. Particularly in disease states of the GI tract that are dominated by eosinophils, such as eosinophilic gastritis and esophagitis, it will be interesting to study whether UDCA has the same potential to suppress tissue eosinophilia as reported in our lung inflammation model. This approach might be more amenable to therapeutic intervention as UDCA is available in an oral formulation. In conclusion, our study shows that UDCA has immunomodulatory capacities to suppress eosinophilic inflammation outside the GI tract, via modulation of DC function.

Conflict of interest

None.

Supporting Information

Additional Supporting Information may be found in the online version of this article and in this thesis:

Methods

Mice

Six- to 8-week-old Balb/c and C57bl/6 female mice were obtained from Harlan (Zeist, the Netherlands). Bone marrow was harvested from farnesoid X receptor (FXR)-deficient (14) mice, and OTII lymphocytes (all C57bl/6 background) were obtained from Erasmus MC or UMC Groningen. The mice were housed under specific pathogen free (SPF) conditions at the animal care facility of the Erasmus MC Rotterdam with the approval of the local animal ethics and welfare committee. The bronchial hyperreactivity (BHR) experiments were performed at the VIB, Ghent, Belgium, with the approval of the local animal ethics and welfare committee.

A mouse model of airway inflammation

To induce airway inflammation, we immunized Balb/c mice with ovalbumin (OVA) (10 μg OVA, grade V from Sigma Aldrich, Zwijndrecht, the Netherlands) emulsified in 2 mg $AL(OH)_3$ (OVA/alum) or as a control with phosphate-

buffered saline (PBS) in alum. Six days later on three consecutive days, mice anesthetized with isoflurane were treated with UDCA 30 μg/animal or diluent intratracheally (i.t.). After recovery, they were challenged by exposure to OVA aerosols 10 mg/ml or PBS during 30 min. Twenty-four hours after the last aerosols, the mice were killed using an anesthetic overdose followed by bleeding from the vena inguinalis and bronchoalveolar lavage (BAL). The lungs were snap-frozen in OCT freezing solution (TissueTek, Zoeterwoude, the Netherlands). The mediastinal lymph nodes were collected and stimulated with OVA. Bronchial hyperreactivity was analyzed 24 h after the last aerosols using Flexivent invasive measurement of dynamic resistance (SCIREQ). Mice were anesthetized with urethane, paralyzed using d-tubocurarine, and tracheotomized, followed by mechanical ventilation. Increasing concentrations of methacholine (MCH) (0–100 mg/ml) were nebulized using Aeroneb (SCIREQ). Dynamic resistance and compliance were recorded after a standardized inhalation maneuver given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of MCH. In some experiments, we performed an adoptive transfer of dendritic cell (DCs) to induce asthma. In brief, myeloid DCs were grown for 8 days from the bone marrow (Balb/c or in case of WT and FxR-KO C57bl/6) in recombinant murine granulocyte macrophage–colony-stimulating factor (rmGMCSF) 20 ng/ml, were subsequently pulsed with OVA or OVA in the presence of UDCA or PBS, and then $1x10^6$ DCs were injected (i.t.). After this treatment, mice were given three additional OVA aerosol challenges.

Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was performed, and cells were collected for cellular differentiation by flow cytometry using a method described elsewhere (15). Antibodies for CD3 labeled with $PeCy^5$ (clone 145-2C11; BD Biosciences, Breda, the Netherlands), CD19 labeled with PeCy5 (clone 1D3; eBiosciences, Vienna, Austria), CD11c labeled with $PeCy^7$ (clone N418; eBiosciences), MHCII labeled with Fitc (M5/114.15.2; eBiosciences), and CCR3 labeled with APC (clone FAB729A; R and D systems, Abingdon, UK). In some experiments, when C57bl/6 animals were used, half of the BAL cells were stimulated with ionomycin (Sigma), phorbol 12-myristate 13- acetate (PMA; Sigma), and Golgiplug (BD Biosciences); incubated at 37°C for 4 h; and subsequently stained with the following antibodies: CD3 APC cy^7 (clone 145-2C11; BD Biosciences), CD4 $PeCy^5$ RM4-5 (eBiosciences), interleukin (IL)-4 (clone 11B11; BD Biosciences), and interferon (IFN)-γ (clone XMG1.2; BD Biosciences). Fixable Aqua Dead Cell Stain kit for 405 nm (Invitrogen, Molecular Probes, Life Technologies Europe BV, Bleiswijk, the Netherlands) was used as a live–dead marker. Cells were measured on a Flowcytometer LSRII (BD Biosciences) and analyzed with FLOWJO software. Bronchoalveolar lavage fluid and supernatants of the OVA-stimulated lymph nodes (LN) cell suspensions were assayed with enzyme-linked immunosorbent assay (ELISA) for the quantification of IL-4, IL-5, IL-10, IL-13, and IFN-γ. T-cell stimulation and eosinophil culture Lymphocytes obtained from animals that were sensitized against OVA were stimulated in vitro in a nonspecific way with culture plate–coated antibodies against CD3 (overnight 1 μg/ml) and antibodies against CD28 in solution (2.5 μg/ml) for 4 days. Ursodeoxycholic acid was present during the whole culture period. To generate eosinophils, bone marrow cells ($0.2x10^6$/well) were cultured in 30% fetal bovine serum (FBS) and IL-5 25 ng/ml, as described (15). Ursodeoxycholic acid was added on day 5. Twenty-four hours later, cells were counted and stained for the eosinophil-specific marker CCR3 (eotaxin receptor) and for live–dead with PI.

Bone-marrow-derived myeloid DCs

To generate mDCs, bone marrow cells were collected from naive mice and cultured in 5% FBS and rmGM-CSF (kindly provided by Dr. K. Thielemans, Belgium) for 9 days as described (16). At day 9, they were exposed to a different concentration of UDCA and pulsed in vitro overnight with 100 μg/ml OVA or sham-pulsed with PBS. Supernatants of DC medium were stored for IL-10 and IL-12 ELISA (BD Biosciences). Visualization of DC/T cell interaction For visualizing dynamic interactions between DCs and specific T cells, OVA-specific T cells obtained from OTII TCR Tg (H-2b) mice were co-cultured with unpulsed bone marrow derived dendritic cells (BMDCs), OVA protein–pulsed BMDCs (100 μg/ml), or OVA peptide (323–339)–pulsed BMDCs (1 μg/ml) treated or not treated with 10 μM UDCA overnight. Prior to incubation, DCs were stained with an fluorescein isothiocyanate (FITC)-labeled antibody to I-Ak,b (M5/114), and T cells were stained with biotin-tagged TCR Vα2 antibodies, followed by Qdot 655 streptavidin conjugates (Invitrogen) essentially as described (17). Stained DCs adhere in a poly-L-lysine (PLL)-coated imaging chamber at 37°C in an atmosphere of 5% CO2. After 1h, T cells were added in a ratio of 1 DC to 10 T cells. Interactions were visualized with an LSM 510 confocal microscope (Zeiss, Sliedrecht, the Netherlands) equipped with 488- and 633-nm lasers. Time-lapse images were collected every 20 s for 40 min. Interactions were quantified using IMARIS software (version 5.0; Bitplane AG, Zurich, Switzerland) (17).

Statistical analysis

For statistical analysis, Kruskal–Wallis one-way ANOVA was used to calculate the overall P-value. A P-value of <0.05 was considered a significant difference between groups. The nonparametric Mann–Whitney U-test was performed to analyze each group with respect to each other. The Pearson correlation test was used to indicate a significant concentration dependency.

Recent findings

An interesting finding was published by Kollerov (36) showing that various fungi causing lung diseases are secreting bile acids such as DCA and UDCA. It could be interesting to investigate if these fungi reduce inflammation to itself by release of UDCA in the lung and are thereby capable to cause fungal infections. Presence of fungi in the lung can act as alarmins enhancing asthmatic responses (37).

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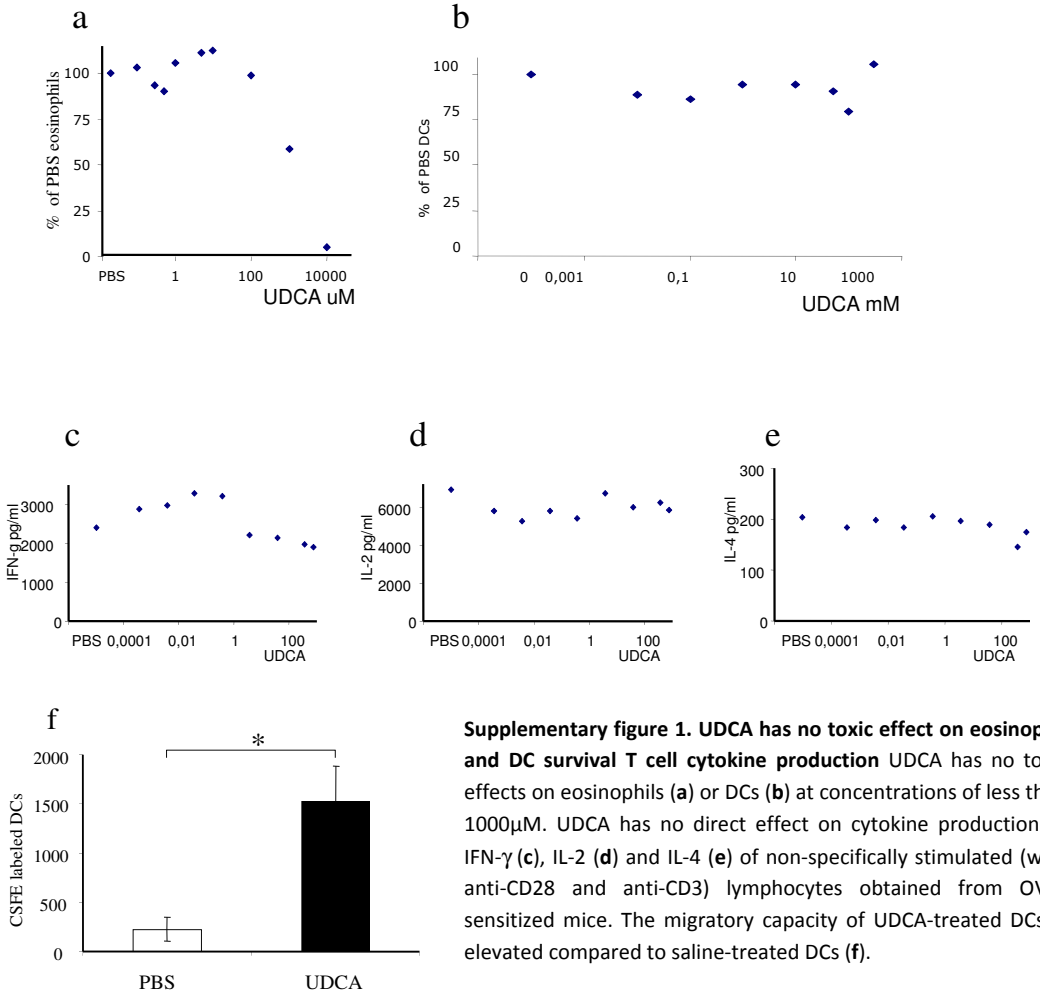
Supporting material

UDCA is not cytotoxic for eosinophils

UDCA treatment reduces eosinophilic airway inflammation, however without affecting the major eosinotrophic cytokine IL-5 or the production of the eosinophil specific chemokine eotaxin. To exclude the possibility of a direct cytotoxic effect of UDCA on eosinophils, that could cause the reduction in eosinophils in UDCA-treated group, we grew eosinophils *in vitro* and exposed them to varying concentrations of UDCA. Bone marrow cells were isolated from mice and cultured in the presence of IL-5 to promote eosinophil differentiation. On day 5, these cells were treated with UDCA and analyzed the next day. The viability of eosinophils, identified as CCR3-positive cells with high scatter characteristics, was analyzed using propidium iodide (PI) exclusion staining. No toxic effect was measured in eosinophils up to a concentration of 1000 µM UDCA (390 µg/ml) (**supplementary figure 1a**, indicating the percentage of total live eosinophils in culture), which is the dose that was given i.t. in the previous in vivo experiments. Higher UDCA concentrations induced eosinophil death. Thus, we applied non-toxic UDCA concentrations in our *in vivo* experiments excluding direct cytotoxic effects of UDCA on eosinophils.



Supplementary figure 1. UDCA has no toxic effect on eosinophil and DC survival T cell cytokine production UDCA has no toxic effects on eosinophils (**a**) or DCs (**b**) at concentrations of less than 1000µM. UDCA has no direct effect on cytokine production of IFN-γ (**c**), IL-2 (**d**) and IL-4 (**e**) of non-specifically stimulated (with anti-CD28 and anti-CD3) lymphocytes obtained from OVA-sensitized mice. The migratory capacity of UDCA-treated DCs is elevated compared to saline-treated DCs (**f**).

UDCA does not influence T cells directly

UDCA treatment of OVA-immunized mice during OVA challenge also resulted in a reduced cytokine production that is classically associated with Th_2 responses, e.g. the increased IL-13 concentration. To address whether UDCA directly suppresses the cytokine production of T-cells, we stimulated T-cells from OVA-immunized mice and challenged these cells non-specifically with plate bound anti-CD3 and with anti-CD28 in suspension. **Supplementary figures 1b-d** show that no differences in T-cell cytokine IL-2, IL-4 and IFN-γ production were observed upon increasing UDCA concentrations. Thus, the cytokine production via non-specific T-cell stimulation was not abrogated by UDCA.

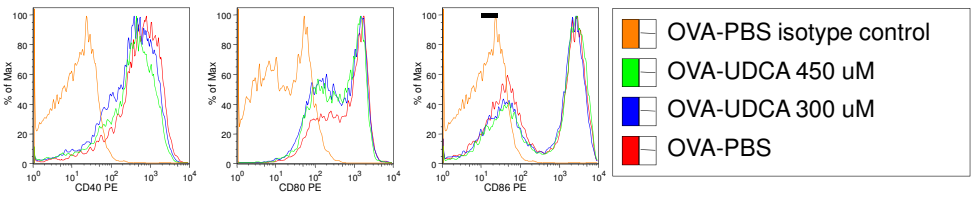
No cytotoxic effect of UDCA on DCs

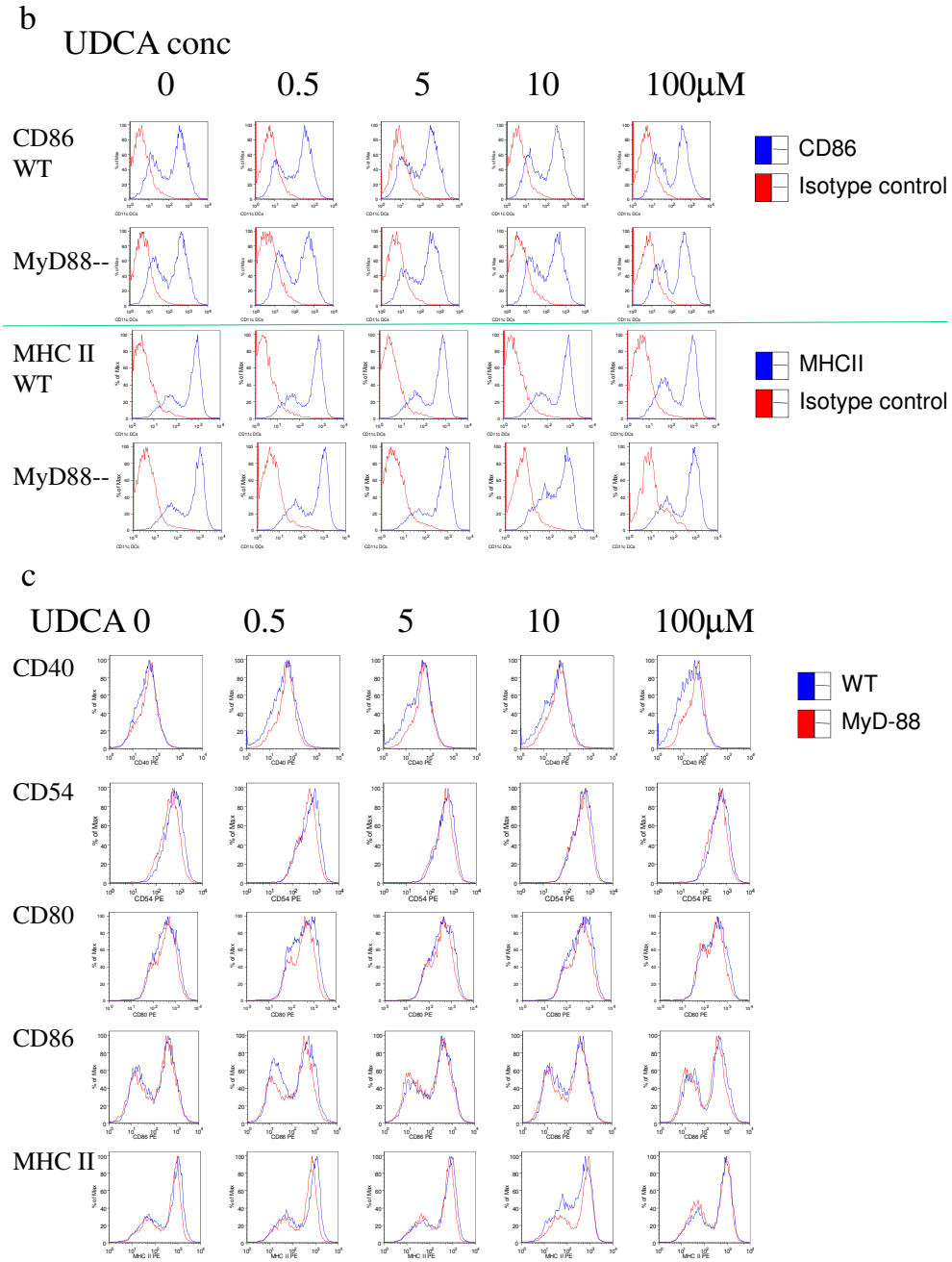
To exclude the possibility of a direct cytotoxic effect of UDCA on DCs, that could cause the reduction in DCs in UDCA-treated group, we grew DCs in vitro and exposed them to UDCA. CD11c-positive GM-CSF-cultured bone marrow cells (bone marrow-derived DCs) were exposed to various UDCA concentrations on day 8 of culture and were subsequently exposed to OVA on day 9 and analyzed on day 10. There was no cytotoxic effect of UDCA on bone marrow DCs (**Supplementary figure 1e**). UDCA-treated DCs have an enhanced migratory activity *in vivo*. Prior overnight incubation with UDCA did not abolish the potential of DCs to migrate from the trachea to the draining mediastinal lymph nodes, yet even enhanced it (**Supplementary figure 1f**), showing the number of labeled DCs reaching the mediastinal nodes 24h after intratracheal injection).

UDCA induces an increased IL-12 production by DCs, promotes induction of IFN-γ in responding T cells while suppressing induction of Th_2 cytokines

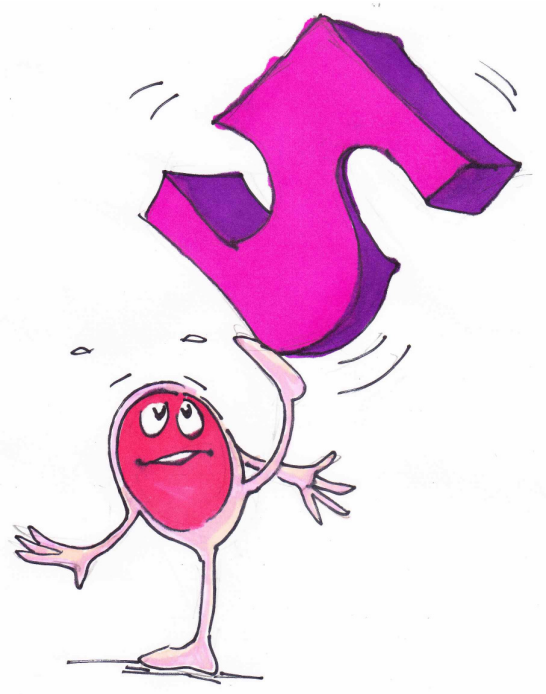
The experiments employing UDCA demonstrated that intratracheal administration of UDCA reduces the number of DCs in allergen challenged lungs. In previous studies, we identified lung DCs as key pro-inflammatory cells that are necessary and sufficient for Th_2 cell stimulation during ongoing airway inflammation (Hammad et al. Nat Rev Imm 2008). CD11c-positive cells (DCs) showed slightly less MHC II staining for UDCA (highest dose) treated DCs than DCs treated with PBS. The phenotype of DCs exposed to UDCA was only slightly less mature (**Supplementary figure 2a**). No significant alteration in costimulatory molecules was observed at different UDCA concentrations (**Supplementary figure 2b**). Activation of bone marrow-derived DCs from MyD88 deficient mice, e.g., mice that lack Toll-like receptor signaling, and their wild-type littermates WT controls with UDCA showed similar results (**Supplementary figure 2c**), confirming that the effects of UDCA are mediated via Toll-like receptor independent mechanism.

a





Supplementary figure 2 UDCA doesn't affect DC phenotype. CD11c-positive cells (DCs) showed slightly less (but not significantly less) MHC II staining (MFI and percentages) for UDCA-treated OVA-pulsed DCs than OVA-pulsed DCs treated with PBS. DCs showed only a slightly less mature phenotype subsequent to exposure to UDCA. The expression of CD40, CD80 and CD86 does not differ between OVA-pulsed DCs that are sham treated or those treated with 300µM or 450µM of UDCA (**a**). No significant alteration in costimulatory molecules was observed at different UDCA concentrations. Bone-marrow-derived DCs from MyD88 (in the absence of signalling from Toll-like receptors) and WT controls showed similar results for MHCII and isotype and CD86 and isotype (**b**), which confirms that the effect of UDCA is independent of Toll-like receptors. There is no difference between WT and MyD88 bone-marrow-derived DCs regarding co-stimulatory molecules (**c**).



An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma

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Uric acid is found to be increased in various inflammatory disorders. Previously, we have shown that uric acid crystals (formed at high levels of uric acid) have adjuvant properties enhancing Th_2 responses. Here we present for the first time that uric acid is released in the lungs upon allergen challenge favouring a Th_2 response. Uricase treatment reduces characteristics of asthma.

An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma

Summary

Although deposition of uric acid (UA) crystals is known as the cause of gout, it is unclear whether UA plays a role in other inflammatory diseases. We here have shown that UA is released in the airways of allergen-challenged asthmatic patients and mice, where it was necessary for mounting T helper 2 (Th_2) cell immunity, airway eosinophilia, and bronchial hyperreactivity to inhaled harmless proteins and clinically relevant house dust mite allergen. Conversely, administration of UA crystals together with protein antigen was sufficient to promote Th_2 cell immunity and features of asthma. The adjuvant effects of UA did not require the inflammasome (Nlrp3, Pycard) or the interleukin-1 (Myd88, IL-1r) axis. UA crystals promoted Th_2 cell immunity by activating dendritic cells through spleen tyrosine kinase and PI3-kinase δ signaling. These findings provide further molecular insight into Th_2 cell development and identify UA as an essential initiator and amplifier of allergic inflammation.

Introduction

Allergic asthma is caused by an inappropriate adaptive T helper 2 (Th_2) cell-mediated immune response to innocuous antigen, leading to eosinophilic airway inflammation, mucus hypersecretion, structural changes to the airway wall, and variable airway obstruction (Barnes, 2008). The cytokines produced by allergen-specific Th_2 cell-type lymphocytes are held largely responsible for orchestrating the many features of asthma. Sensitization results from allergen recognition by structural cells of the airways and innate immune cells like basophils and dendritic cells (DCs) that induce and amplify adaptive Th_2 cell-mediated immune responses (Barrett and Austen, 2009; Hammad et al., 2009, 2010; Lambrecht and Hammad, 2009; Sokol et al., 2008). The precise molecular mechanisms involved in allergenicity and Th_2 cell response induction by DCs are incompletely understood (Paul and Zhu, 2010). Allergens have a complex molecular structure and often display enzymatic activity that stimulates epithelial and innate immune cells through protease-activated receptors (Hammad and Lambrecht, 2008; Sokol et al., 2008; Tang et al., 2010), whereas others like house dust mite (HDM) contain endotoxin or other pathogen-associated microbial patterns (PAMPS) that trigger epithelial cells and/or DCs in a toll-like receptor 4 (TLR4)- or C-type lectin receptor-dependent manner (Hammad et al., 2009; Nathan et al., 2009; Trompette et al., 2009). Triggering of PAMP receptors on epithelial cells and/or basophils subsequently leads to release of innate pro- Th_2 cell cytokines like thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-25, and IL-33 that instruct DCs to promote Th_2 cell-type immunity in the mediastinal lymph nodes (Hammad and Lambrecht, 2008;Hammad et al., 2009, 2010; Rank et al., 2009; Sokol et al., 2008; Zhou et al., 2005). Although contamination of allergens with PAMPs that stimulate pattern recognition receptors provides a good explanation for how immunity can be induced to harmless allergens, another could be that allergens trigger release of damage-associated molecular patterns (DAMPs) that occur intracellularly in homeostasis but are

released extracellularly upon physical or metabolic stress (Matzinger, 2002). A well-known DAMP is uric acid (UA) released from dying or stressed cells (Shi et al., 2003; Vorbach et al., 2003). Uric acid crystals potently trigger acute neutrophilic inflammation through stimulation of the NLRP3 inflammasome and release of interleukin-1β (Chen et al., 2006; Martinon et al., 2006). This pathway is at the heart of gouty inflammation, as supported by the absence of UA-induced neutrophilic inflammation in mice lacking the NLRP3, ASC (Pycard), IL-1R, or its downstream adaptor MyD88 and by the success of IL-1 receptor antagonist (IL-1RA) treatment for gout (So et al., 2007; Ghosh et al., 2013). We have reported recently that UA is released in the peritoneal cavity after injection of the Th_2 cell adjuvant aluminum hydroxide or alum, commonly co- injected with harmless antigens intraperitoneally to induce allergic sensitization and asthma (Kool et al., 2008b). In addition to inducing release of UA, alum can also directly trigger the NLRP3 inflammasome and stimulate secretion of bioactive IL-1β (Eisenbarth et al., 2008; Kool et al., 2008a; Li et al., 2008; McKee et al., 2009). These studies have aroused interest in the UA-inflammasome-IL-1 axis as a trigger of Th_2 cell development. Here, we hypothesized that uric acid is a general inducer and amplifier of Th_2 cell immunity. By using models of intraperitoneal injection, as well as airway exposure of HDM allergen in mice and patients, we found that UA is present at sites of Th_2 cell development, where it is necessary and sufficient for induction of Th_2 cell immunity. Contrary to expectation, this Th_2 cell adjuvant effect did not require triggering of the NLRP3-ASC complex, nor signaling through the IL-1R. Rather, UA induced Th_2 cell immunity by triggering DC activation in a spleen tyrosine kinase (Syk)- and PI3-kinase δ-dependent manner. These findings identify UA as an unexpected initiator and amplifier of Th_2 cell immunity and allergic inflammation.

Results

Uric Acid Is Necessary and Sufficient for Induction of Th_2 Cell Immunity to Harmless Antigen

We have reported that UA is released in the peritoneal cavity after injection of alum (Kool et al., 2008b) and is a necessary intermediate for early DC activation *in vivo*, whereas others argued that the immunostimulatory effects of alum did not require UA (Franchi and Nunez, 2008). As shown in **Figure 1A**, the degradation of UA by uricase treatment reduced secretion of bioactive IL-1β into the peritoneal cavity, whereas production of IL-1β by DCs *in vitro* did not require endogenous release of UA. Therefore, the important role of UA in mediating the adjuvant activity of alum is mainly revealed *in vivo*. The increased UA production after injection of OVA in alum was found to be intact in *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Casp1*$^{-/-}$ mice, excluding an important role for the TLR4 or inflammasome-IL1β axis in its induction (**Figure S1** available online). Because alum is frequently used to induce allergy in animal models of asthma, we hypothesized that UA is necessary for promoting adaptive Th_2 cell immunity to OVA+alum. Injection of OVA+alum into BALB/c mice led to an increased concentration of UA in the peritoneal cavity, which could be neutralized by treatment with uricase (**Figure 1B**). Uricase treatment during priming to OVA+alum eliminated Th_2 cell-dependent bronchoalveolar lavage (BAL) fluid eosinophilia (**Figure 1C**), peribronchial inflammation and goblet cell hyperplasia (**Figure 1D**), secretion of IL-5 in the BAL fluid (**Figure 1E**), production of IL-4, IL-5, IL-10, and IL-13 (but not IFN-g) in mediastinal LN (MLN) cultures restimulated with OVA antigen for 3 days *ex vivo* (**Figure 1F**), and production of serum OVA-specific IgE and IgG_1 (**Figure 1G**). There was also a trend toward reduced production of IgG_{2a} by uricase treatment. OVA+alum injection also

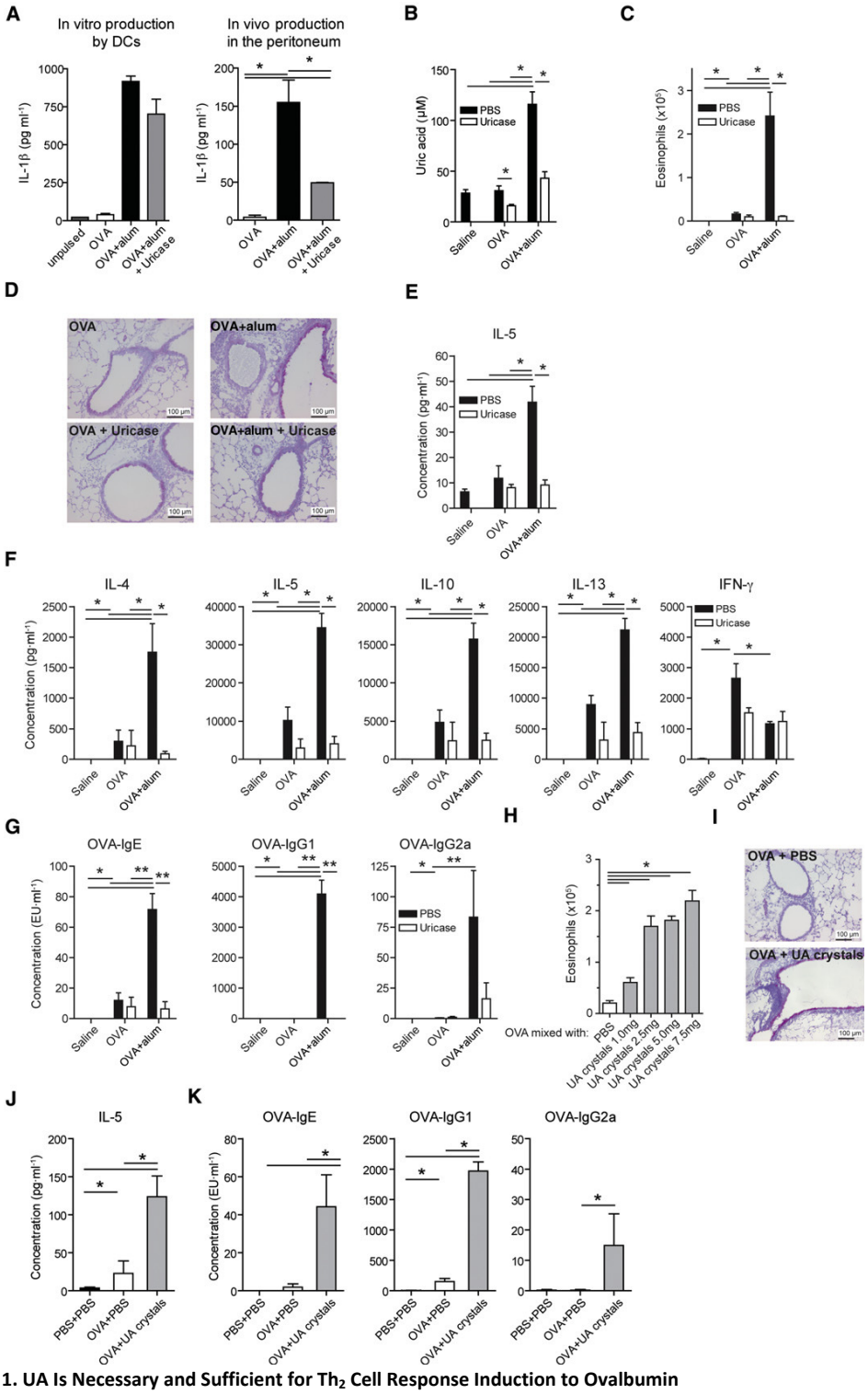

Figure 1. UA Is Necessary and Sufficient for Th_2 Cell Response Induction to Ovalbumin

(**A**) In vitro bone marrow-derived GM-CSF-cultured DCs were stimulated with OVA or OVA+alum in the presence or absence of uricase (0.1 U/ml). *In vivo*, OVA or OVA+alum with or without 50U uricase was injected intraperitoneally (i.p.). Bioactive IL-1β was measured in the supernatant or the peritoneal lavage 2 hr after exposure. (**B**) UA in the peritoneal lavage 2 hr after injection of OVA or OVA+alum in mice treated or not with uricase. (**C**) BALB/c mice were treated with PBS or uricase 5 min before sensitization with OVA or OVA+alum i.p. on day 0. On day 7, mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17–19. On day 20, BAL was analyzed for the presence of eosinophils. (**D**) Lung sections were stained with periodic acid Schiff (PAS) reagent to visualize goblet

cell metaplasia. Scale bars represent 100 µm. (**E**) IL-5 concentration in BAL supernatants of mice treated as described in (C). (**F**) Cytokine production of *in vitro* restimulated MLN cells taken on day 20 and cultured for 3 days in vitro in the presence of OVA. (**G**) Serum OVA-specific Igs were determined by ELISA. (**H**) Mice were sensitized with OVA or OVA mixed with different doses of UA crystals i.p. on day 0. On day 7, all mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17–19. On day 20, the number of eosinophils was assessed in BAL. (**I**) Lung sections stained with PAS. Scale bar represents 100 mm. (**J**) IL-5 in BAL supernatant. (**K**) Serum OVA-specific Igs determined by ELISA. Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, n = 4–8 mice/group. Experiments were repeated twice; data shown are representative of all experiments.

led to release of extracellular ATP, another DAMP known to activate the NLRP3 inflammasome via the $P2X_7$ receptor (Mariathasan et al., 2006) and promote Th_2 cell immunity (Idzko et al., 2007), yet its neutralization or use of $P2X_7R^{-/-}$ mice did not reveal a contribution to Th_2 cell immunity (**Figure S2**). To address whether UA was also sufficient to induce Th_2 cell immunity, we injected OVA mixed with UA crystals. Compared with control mice receiving OVA, this treatment dose dependently increased airway eosinophilia (**Figures 1H and 1I**), goblet cell hyperplasia (**Figure 1I**), IL-5 in BAL fluid (**Figure 1J**), and production of OVA- specific IgE, IgG_1, and IgG_{2a} (**Figure 1K**). Injection of OVA+UA also led to increased production of IL-4, IL-5, and IL-10, but not IFN-γ, in MLN cells (data not shown). These responses were of similar strength as those induced by injection of OVA+alum (**Figures 1C–1E**). Therefore, UA is necessary and sufficient for Th_2 cell response development to intraperitoneal injection of harmless antigen, identifying it as a potent Th_2 cell adjuvant.

Endogenous UA Is Necessary for Development of Th2 Cell Immunity to HDM Allergen in the Absence of Adjuvant

We next investigated the involvement of endogenous UA in response to complex and relevant allergens in the airways. A single intranasal HDM administration in naive mice resulted in a rapid increase in UA concentration in BAL fluid and lung (**Figure 2A**) compared with PBS. Mice sensitized and challenged with HDM developed features of allergic asthma, like BAL fluid and lung eosinophilia, lymphocytosis, goblet cell hyperplasia (**Figures 2B and 2D**), and airway hyperresponsiveness (AHR) to increasing doses of methacholine (**Figure 2C**). Intratracheal administration of uricase on day 0 at the time of sensitization neutralized the increase in UA (**Figure 2A**) and strongly reduced airway lymphocytosis and eosinophilia and AHR (**Figures 2A–2D**). To explain the mechanism of action, we evaluated the production of cytokines known to promote eosinophilic inflammation and Th_2 cell immunity (Hammad and Lambrecht, 2008; Saenz et al., 2008). As already reported, the concentration of GM-CSF, TSLP, and IL-25 (**Figure 2E**) were increased in the lungs of animals exposed to a single HDM injection, compared with PBS (Hammad et al., 2009). However, this innate cytokine response was severely reduced in mice treated with uricase. House dust mite-driven Th_2 cell development relies on antigen presentation by inflammatory $Ly6C^{hi}CD11b^{+}$ DCs that take up antigen in the lung and migrate to the MLN (Hammad et al., 2010). To address antigen uptake and migration of these inflammatory DCs, we injected fluorescent OVA together with HDM allergen. In mice injected with OVA+HDM, migratory DCs carrying fluorescent cargo were easily identified in the MLN. After uricase treatment, this migration was strongly reduced (**Figure 2F**). We next searched for the source of increased UA production. Within 2 hr after HDM administration, there was increased immunoreactivity for UA in airway epithelial cells and major histocompatibility complex II (MHCII) positive alveolar macrophages and recruited monocytes (**Figures 2G**)

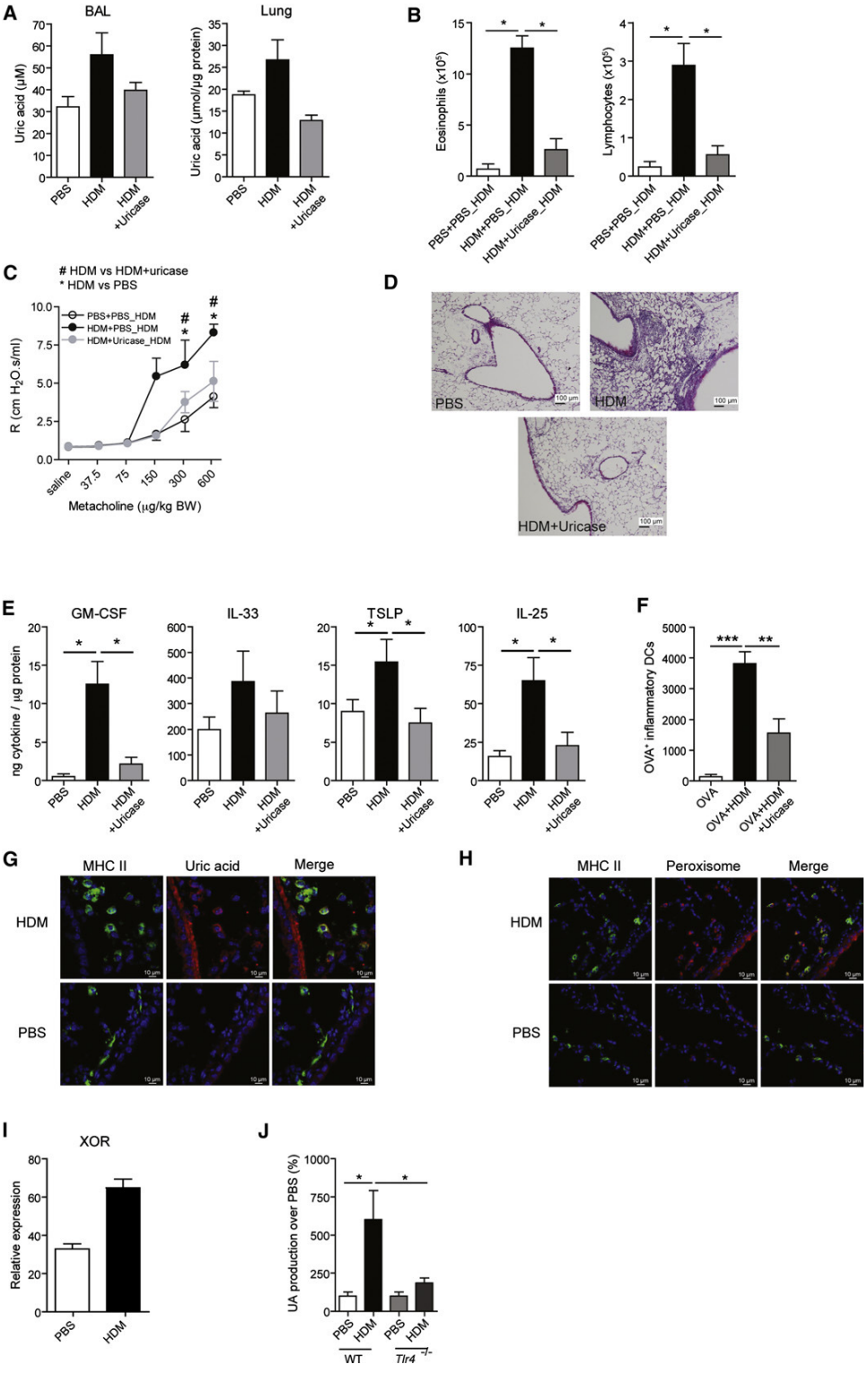

Figure 2. Uric Acid Plays a Key Role in Sensitization to House Dust Mite Allergen via the Airways

(**A**) C57BL/6 mice were sensitized i.n. with PBS or HDM and treated or not with uricase, and UA concentration was determined 2 hr later in the BAL and lung homogenates. (**B**) Mice were sensitized i.n. on day 0 with PBS, HDM, or HDM+uricase and challenged i.n. with HDM on days 7–11. On day 14, BAL was examined for the presence of eosinophils and lymphocytes. (**C**) Lung function, as determined by invasive measurement of airway resistance (R) in response to increasing concentrations of methacholine. #p < 0.05 compared with untreated, HDM-sensitized group;

*p < 0.05 compared with nonsensitized group. (**D**) PAS staining of lung sections. Scale bars represent 100 μm. (**E**) Production of GM-CSF, IL-33, TSLP, and IL-25 in lung homogenates of mice 2 hr after the i.n. administration of PBS, HDM, or HDM+uricase. (**F**) Antigen uptake and migration of inflammatory $CD11b^{+}Ly6C^{+}$ DCs to the mediastinal LN, 24 hr after intranasal injection of OVA-AF647 in the presence or absence of HDM, and effect of treatment with uricase. ***p < 0.005 compared with OVA; **p < 0.05 compared with OVA+HDM. (**G**) Fluorescence staining of uric acid (red) and MHCII (green) in lungs of animals exposed for 2 hr to HDM or PBS. Nuclei were counterstained with DAPI (blue). Scale bars represent 10 μm. (**H**) Fluorescence staining of the peroxisomal marker PMP70 (red) and MHCII (green) in lungs of animals exposed for 2 hr to HDM or PBS. Nuclei were counterstained with DAPI (blue). Scale bars represent 20 μm. (**I**) Expression of XOR mRNA normalized for HPRT by real-time quantitative RT-PCR. (**J**) Increased production of UA depends on TLR4 triggering by HDM. In this experiment C57BL/6 WT or $Tlr4^{-/-}$ mice received HDM, and UA concentration was measured 2 hr later. Data are shown as mean ± SEM, *p < 0.05, n = 4–8 mice/group. Experiments were repeated twice. Data shown are representative of all experiments.

compared to mice administered PBS. Strong UA immunoreactivity was seen along the basement membrane of HDM-exposed mice. Although UA release occurs in response to necrotic cell death, UA is also produced as a pulmonary defense mechanism to oxidative damage, through increased xanthine oxidoreductase (XOR) enzyme activity inside peroxisomes (Vorbach et al., 2003). In bronchial epithelial cells and MHCII-positive cells, HDM exposure induced enhanced staining for the 70 kDa peroxisomal marker PMP70, suggestive of peroxisomal biogenesis (**Figure 2H**; Imanaka et al., 1999). The expression of XOR was increased in BAL cells of HDM-exposed mice 2 hr after instillation compared with PBS (**Figure 2I**). We (Hammad et al., 2009) and others (Phipps et al., 2009; Trompette et al., 2009) recently identified TLR4 signaling as a crucial early trigger for the innate pro-Th_2 cell immune response to allergens. In contrast to the alum-induced increase of UA in the peritoneal lavage (**Figure S1**), HDM-driven production of UA was severely reduced in C57BL/6 *Tlr4*$^{-/-}$ mice (**Figure 2J**), suggesting that triggering of TLR4 by HDM is necessary for activation of XOR and UA production in the airways in response to allergens.

Uric Acid Is Increased in Allergen-Challenged Human Asthmatics and Mice

To study whether our findings had any bearing on human asthma, BAL fluid was collected from 21 asthmatic subjects, 10 min and 24 hr after segmental challenge with saline or specific allergens (rye, birch, or HDM). There was no difference in the concentration of UA between saline- and allergen-challenged segments after 10 min. In contrast, 24 hr after allergen provocation, the UA concentration was markedly elevated in the BAL fluid from allergen- compared to saline-challenged segments (**Figure 3A**). This increase in UA concentration correlated with the influx of eosinophils and lymphocytes into the BAL (**Figures 3B and 3C**). Subgroup analysis of patients showing a minor rise in UA showed that this was not correlated with the type of allergen used nor the concentration of IgE in the serum, but rather on maintenance inhaled corticosteroid (ICS) therapy up to 7 days prior to allergen challenge (**Figure S3**). To test whether UA neutralization would have any therapeutic potential, we again turned to the mouse model of HDM-induced asthma. Mice were first sensitized to HDM and were treated with uricase prior to each allergen challenge. HDM challenge induced a rise in UA in BAL fluid, still measurable 3 days after the last of five challenges (**Figure 3D**), and uricase treatment during challenge reduced airway eosinophilia (**Figure 3E**), methacholine-induced AHR, and IL-13 concentration in the BAL fluid (**Figures 3F and 3G**). Allergen challenge induced high numbers of eosinophils in the BAL fluid, which indicates that cellular death of recruited eosinophils could be a trigger for UA production. Uricase treatment had similar effects in a

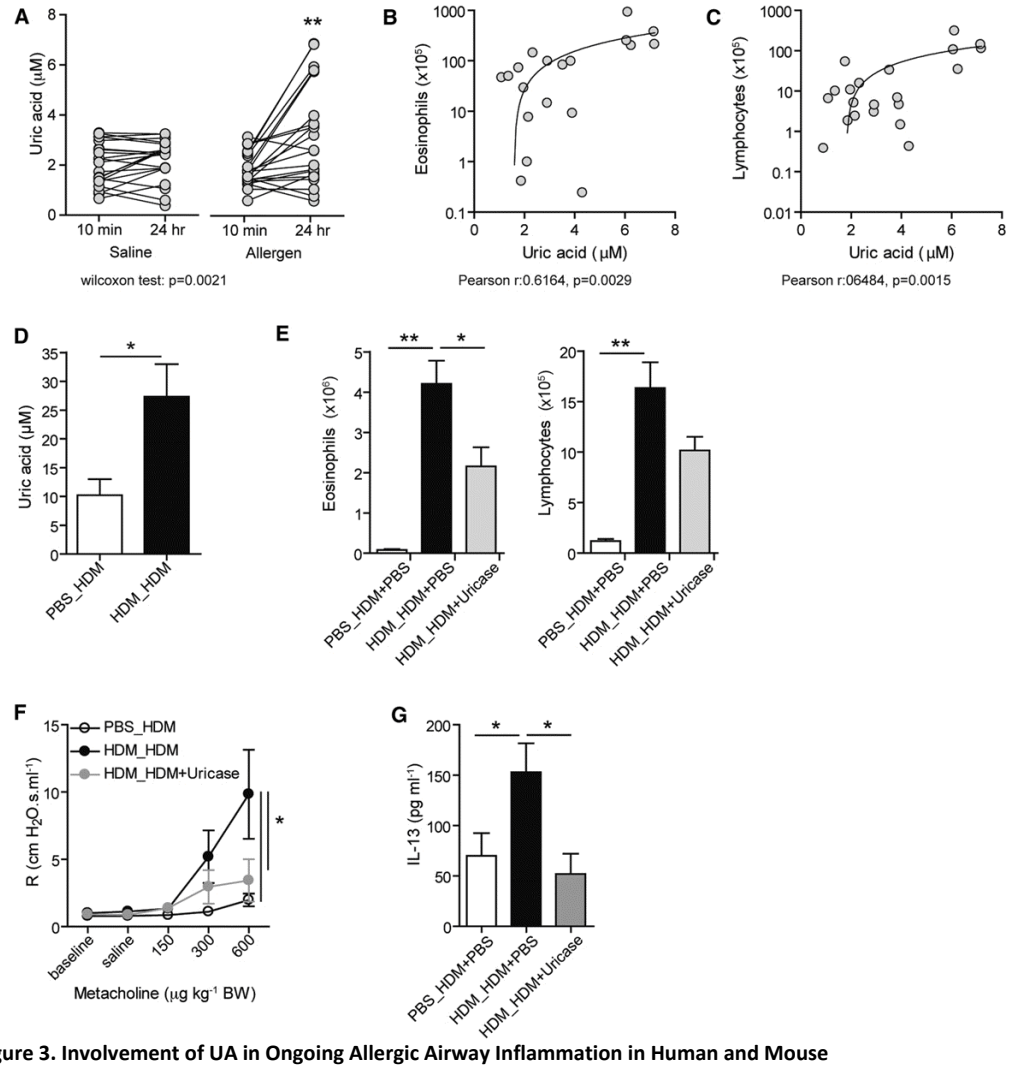

Figure 3. Involvement of UA in Ongoing Allergic Airway Inflammation in Human and Mouse

(**A**) UA concentration in BAL of asthmatic patients 10 min or 24 hr after segmental bronchial allergen or saline challenge. (**B** and **C**) Correlation of UA concentration with the number of BAL eosinophils (**B**) or lymphocytes (**C**) 24 hr after allergen challenge. (**D**) Concentration of UA in BAL 3 days after the last of five HDM challenges in HDM-sensitized mice. (**E**) Treatment of HDM-sensitized mice with uricase (10 U) concurrent with each allergen challenge reduced airway eosinophilia, measured 3 days after the last challenge. (**F**) Effect of uricase treatment prior to allergen challenge on AHR to increasing doses of metacholine. (**G**) Concentration of IL-13, a cytokine associated with induction of AHR, in BAL. Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, n = 4–8 mice/group. Experiments were repeated twice. Data shown are representative of all experiments.

milder sensitization model in which only 20% of the number of eosinophils was recruited compared with the high dose sensitization model (**Figure S4**), arguing against the idea that UA plays a role only when there would be massive cellular recruitment and death.

UA Crystals, Alum, and HDM Promote Th_2 Cell Immunity in the Absence of NLRP3 Inflammasome Activation

Although UA is the prototypical activator of the NLRP3 inflammasome and several groups (Eisenbarth et al., 2008; Franchi and Nunez, 2008; Hornung et al., 2008; Kool et al., 2008a; McKee et al., 2009) have shown that alum directly triggers NLRP3 *in vitro*, there is considerable controversy whether the inflammasome-IL-1β pathway is necessary for adjuvanticity *in vivo*

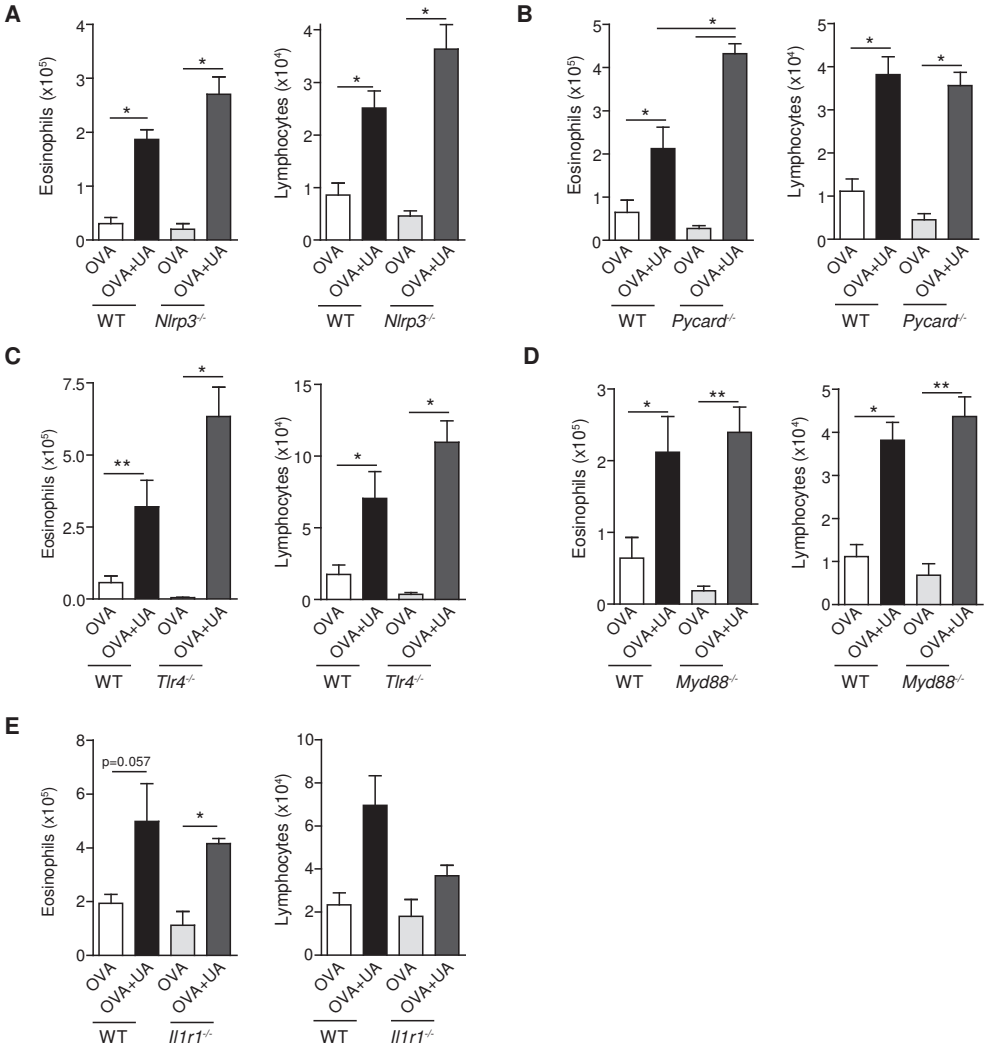

Figure 4. Uric Acid-Mediated Adaptive Th2 Cell Responses Do Not Require the IL-1-Inflammasome Axis C57BL/6 mice were administered OVA mixed or not with UA crystals (UA) on day 0, boosted on day 7 with OVA intraperitoneally (i.p.), and challenged with OVA aerosols on days 17–19. (A) Cellular composition in the BAL of WT and *Nlrp3*$^{-/-}$ mice. (B) Cellular composition in the BAL fluid (BALf) of WT and *Pycard*$^{-/-}$ mice. (C) Cellular composition in the BALf of WT and Tlr4$^{-/-}$ mice. (D) Cellular composition in the BALf of WT and *Myd88*$^{-/-}$ mice. (E) Cellular composition in the BAL of WT and *Il-1r*$^{-/-}$ mice. Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, n = 4–6 mice/group. Experiments were repeated 2–3 times. Data shown are representative of all experiments.

(Franchi and Nunez, 2008; Williams et al., 2010). We therefore studied the involvement of this pathway in UA-driven Th_2 cell immunity *in vivo*. Wild-type C57BL/6 mice immunized with OVA+UA showed a marked increase in eosinophilia and lymphocytosis in BAL (**Figures 4A–4E**), and this response was not affected in *Nlrp3*$^{-/-}$ or *Pycard*$^{-/-}$ mice, lacking essential components of the inflammasome complex (Martinon et al., 2006). Because there might be inflammasome-independent pathways to generate IL-1β, we also immunized mice lacking the IL-1R or mice lacking the critical IL-1R signaling intermediate MyD88. It has also been suggested that UA crystals signal via TLR4, although this could be due to contaminating endotoxin. Strikingly, *Il1r*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice all developed normal Th_2 cell immunity to OVA+UA (**Figures 4C– 4E**). We also studied whether development of Th_2 cell immunity and asthma in response to OVA+alum relied on Nlrp3. Contrary to a recent report (Eisenbarth et al., 2008), we observed

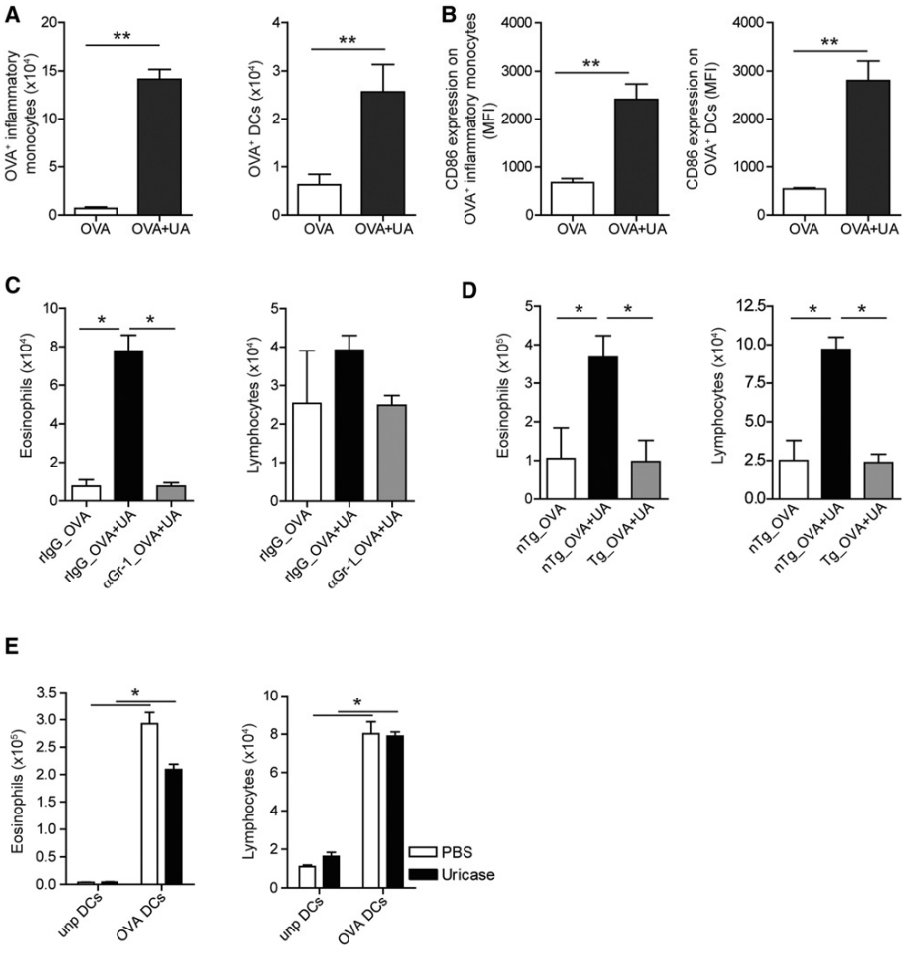

Figure 5. Inflammatory Monocytes and Dendritic Cells Are Critical for the Uric Acid-Mediated Adaptive Th_2 Cell Responses (**A**) C57BL/6 mice received OVA-AF647 mixed or not with UA crystals intraperitoneally (i.p.), and 24 hr later the peritoneal lavage was examined for the presence of $OVA^+CD11b^+Ly6C^{hi}$ inflammatory monocytes and DCs. (**B**) OVA^+ inflammatory monocytes and DCs were analyzed for the expression of CD86. (**C**) C57BL/6 mice were treated with Gr-1 or control antibodies (500 μg/mouse) on day -1, 0, 1, 2. On day 0, mice were treated with OVA mixed or not with UA crystals (UA), boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BALf is shown. (**D**) C57BL/6 *Cd11c*-DTR mice were treated with diphtheria toxin (100 ng/mouse i.p.) on day -1. On day 0, mice were treated with OVA mixed or not with UA crystals, boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BALf was determined by flow cytometry. (**E**) BALB/c mice were sensitized by intratracheal instillation of 1 x 10^6 unpulsed or OVA-pulsed bone marrow-derived GM-CSF DCs with or without uricase. On days 10–12, mice were challenged with OVA aerosols. Cellular composition in the BAL is shown. Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, n = 4–6 mice/group. Experiments were repeated 2–3 times. Data shown are representative of all experiments.

that Th_2 cell-dependent asthmatic airway inflammation developed normally in *Nlrp3*$^{-/-}$ mice immunized with OVA+alum (**Figure S5**). To address whether HDM-driven asthma was dependent on these pathways, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, and *P2X*$_7$*R*$^{-/-}$ mice were sensitized and challenged with HDM via the airways but showed no reduction in Th_2 cell-dependent eosinophilia (**Figure S6**). As already reported, *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$ mice had reduced HDM-driven airway inflammation (data not shown; Hammad et al., 2009, 2010). In conclusion, the

prototypical Th_2 cell stimuli alum and HDM, like UA crystals, do not rely on the NLRP3 inflammasome to induce Th_2 cell adaptive immunity leading to allergic airway inflammation.

UA Crystals Recruit Inflammatory DCs that Are Necessary and Sufficient for Th_2 Cell Immunity

Twenty-four hours after injection of OVA-Alexa Fluor 647+UA, there was a strong increase in the number of inflammatory $Ly6C^{hi}CD11b^{+}$ monocytes and inflammatory $Ly6C^{+}CD11b^{+}$ DCs that had ingested OVA in the peritoneal cavity compared with mice injected with OVA-AF647 alone (**Figure 5A**), and these recruited cells had higher expression of the co-stimulatory molecule CD86 (**Figure 5B**). Although recruitment and activation of DCs suggests a functional role for these cells in the UA effect, we still wanted to address whether DCs are really necessary for mediating Th_2 cell immunity, because recent work suggested that DCs are poor inducers of Th_2 cell immunity, whereas basophils readily are (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). We therefore injected OVA+UA in *Cd11c*-DTR C57BL/6 mice, together with diphteria toxin (DT) to deplete DCs (Jung et al., 2002). DT injection in Tg mice abolished airway eosinophilia induced by OVA+UA (**Figure 5D**). This effect was also seen when we injected an antibody to Ly6C/G (Gr1) that depletes inflammatory monocytes (**Figure 5C**), but in this setting one cannot exclude that neutrophils play an additional role. To finally demonstrate that inflammatory DCs are sufficient to induce Th_2 cell immunity, we injected GM-CSF-cultured OVA-pulsed DCs (a model to generate inflammatory type DCs) into the peritoneal cavity, followed by OVA challenge to the lung. Intraperitoneal injection of OVA-pulsed DCs, but not unpulsed DCs, was able to prime for an eosinophilic Th_2 cell response (**Figure 5E**). However, treatment with uricase did not inhibit Th_2 cell development induced by DC injection, demonstrating that the immunostimulatory effects of UA are upstream of DC activation *in vivo*. These experiments also demonstrate that uricase treatment does not have a generalized off-target suppressive effect on Th_2 cell polarization, but rather inhibits inflammatory type DC recruitment and activation.

UA Activates DCs and Th_2 Cell Immunity in a Syk- and PI3-Kinase δ-Dependent Manner

The above data implied that DCs are a crucial intermediate cell type in mediating the Th_2 cell immune response to OVA+UA (**Figure 5**) but also that the NLRP3-ASC-IL1R axis were not involved in this process *in vivo* (**Figure 4**). UA crystals mixed with OVA *in vitro* were able to induce the secretion of bioactive cleaved IL-1β in inflammatory DCs, and this effect was completely absent in cells of *Nlrp3*-/- mice, essentially showing that UA does trigger this inflammasome pathway in DCs. However, OVA+UA also induced secretion of IL-6 and TNF-α in inflammatory DCs and these responses were not abolished in *Nlrp3*-/- mice. Strikingly, OVA+UA did not induce production of the Th_1 cell instructive cytokine IL-12. Recently, via atomic force microscopy, UA crystals were shown to directly engage cholesterol-rich cellular membranes of DCs in a receptor-independent manner, leading to recruitment and activation of spleen tyrosine kinase (Syk) on membrane-associated immunoreceptor tyrosine activation motifs (Ng et al., 2008). Other studies have also identified Syk signaling and downstream PI3-kinase activation as an essential intermediate of UA-induced human neutrophil activation (Desaulniers et al., 2001; Popa-Nita et al., 2007). When the *in vitro* Syk inhibitor piceattanol was added, UA+OVA no longer induced IL-1β, IL-6, or TNF-α production in inflammatory DCs,

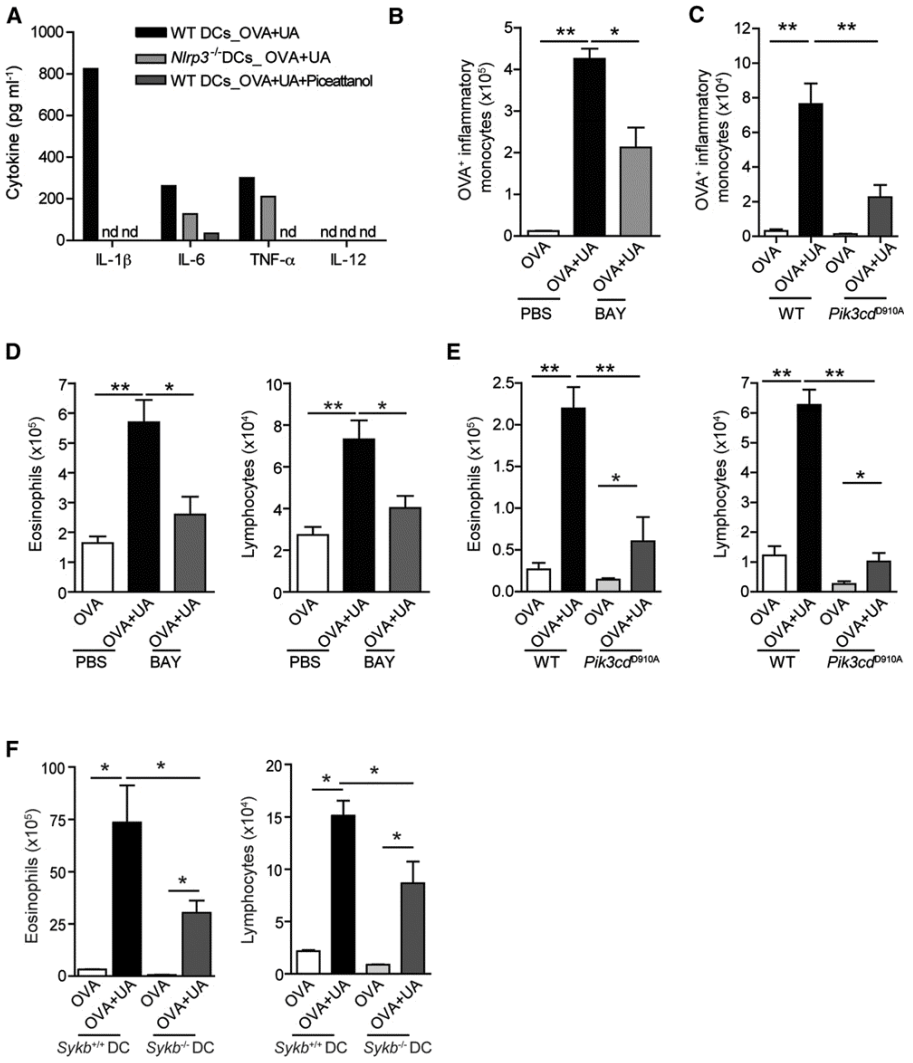

Figure 6. UA Crystals Induce Th_2 Cell Immunity through Spleen Tyrosine Kinase and PI3-Kinase δ (**A**) Bone marrow-derived DCs were cultured in GM-CSF from WT and *Nlrp3*$^{-/-}$ mice and pre-incubated with the Syk inhibitor piceatennol for 30 min (50 mM), then pulsed with OVA (100 μg/ml) with or without UA crystals (250 μg/ml) for 3 hr, after which cytokine concentration was determined in the cell supernatant. (**B**) C57BL/6 mice were treated twice a day on days -1 and -2 orally with the specific Syk inhibitor BAY61-3606 (Yamamoto et al., 2003) and injected on day 0 with OVA-AF647 mixed or not with UA crystals intraperitoneally (i.p.). 24 hr later, the peritoneal lavage was examined for the presence of OVA$^+$ inflammatory monocytes. (**C**) Pik3cdD910A or WT C57BL/6 mice were injected with OVA-AF647 mixed or not with UA crystals (UA) i.p., and 24 hr later the peritoneal lavage was examined for the presence of OVA$^+$ inflammatory monocytes. (**D**) C57BL/6 mice were treated twice a day on days -1 and -2 orally with the specific Syk inhibitor BAY61-3606 . On day 0, mice were treated with OVA mixed or not with UA crystals, boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BAL is shown. (**E**) Cellular composition in the BALf of WT and Pik3cdD910A mice, which have been sensitized with OVA+UA crystals and challenged with OVA aerosol as described in (D), is depicted. (**F**) Cellular composition in the BAL of Sykb$^{-/-}$ DC and Sykb+/+ DC chimeric mice, which were sensitized with OVA+UA crystals and challenged with OVA as described in (D). Data are shown as mean ± SEM, *p < 0.05, **p < 0.01, n = 4–6 mice/group, representative of at least two experiments per panel.

suggesting that this pathway was more broadly involved in inducing cytokine production in DCs (**Figure 6A**). Oral administration of the Syk inhibitor BAY61-3606 reduced influx of OVA-positive inflammatory Ly6C$^+$CD11b$^+$ monocytes in the peritoneum 24 hr later (**Figure 6B**). Signaling downstream of Syk has been shown to involve interaction of p85 with p110d

subunits of PI3-kinase (Popa-Nita et al., 2007). The number of $OVA^{+}Ly6C^{+}CD11b^{+}$ monocytes 24 hr after injection of OVA+UA was strongly reduced in mice expressing a catalytically inactive form of the p110d subunit of PI3-kinase (D910A) (**Figure 6C**). We finally addressed whether Syk and downstream PI3-kinase δ signaling was important for development of adaptive Th_2 cell immunity to OVA+UA. Oral administration of BAY61-3606 compound strongly reduced airway lymphocytosis and eosinophilia (**Figure 6D**). Accordingly, OVA+UA did not induce Th_2 cell immunity in Pik3cdD910A mice (**Figure 6E**), although this finding could also reflect effects on other cells like mast cells (Ali et al., 2004). To address whether the inhibition of Syk was specifically acting at the level of DCs, we took a genetic approach and immunized *Cd11c*-Cre x $Sykb^{fl/fl}$ mice specifically lacking Syk in $CD11c^{hi}$ cells (unpublished observations) or control mice with OVA or OVA+UA crystals. Upon OVA challenge, the degree of reduction in airway eosinophilia in these $Sykb^{-/-}$ DC mice was of similar magnitude as those treated with a pharmacological Syk antagonist, suggesting indeed that Syk triggering in DCs is crucial for mounting Th_2 cell immunity to UA (**Figure 6F**).

Discussion

Although UA is a known trigger for acute neutrophilic inflammation (Martinon et al., 2006), we here identified its role as a promotor of Th_2 cell-dependent allergic inflammation. The release of UA was seen in patients with allergic asthma challenged with relevant allergen, in naive and sensitized mice exposed to the prototypical Th_2 cell inducer and relevant HDM allergen, and after injection of the prototypical Th_2 cell adjuvant alum. The Th_2 cell-inducing capacity of UA occurred independently from its actions on the NLRP3-ASC-IL-1-MyD88 axis. UA is released upon alum administration *in vivo*, but not *in vitro*, so these findings could explain the controversy that has arisen as to whether triggering NLRP3 inflammasome activation is crucial for alum adjuvanticity (Marrack et al., 2009). All authors that have studied the involvement of NLRP3 agree that NLRP3, PYCARD (ASC), and caspase-1 are crucial for alum-induced IL-1β secretion *in vitro* and *in vivo*, but this does not automatically imply that all adjuvant effects of alum must therefore occur via this pathway (Franchi and Nunez, 2008; Li et al., 2007, 2008; McKee et al., 2009). Our findings suggest that UA released upon alum injection *in vivo* is by itself sufficient to induce Th_2 cell adjuvant effects that do not depend on the inflammasome-IL-1R pathway. It has indeed long been known that alum-induced asthma models do not rely on IL-1R signaling (Piggott et al., 2005; Schmitz et al., 2003) nor on the downstream essential adaptor molecule MyD88 (Piggott et al., 2005). One report has suggested that Nlrp3 is absolutely required for alum to induce Th_2 cell immunity to OVA and subsequent development of allergic type inflammation of the lung (Eisenbarth et al., 2008), at odds with findings in the current report. These authors, however, also demonstrated that the effects of alum were MyD88 independent, arguing against a role for IL-1R signaling, given the crucial role of MyD88 as a signaling intermediate of the IL-1R pathway. These differences could be explained best by subtle differences in antigens used, type of alum used, and timing of analysis. Also we have described previously that NLRP3, IL-1, and MyD88 are involved in the early innate response of DCs to alum injection in the peritoneal cavity, but clearly these reactions are redundant with other pathways to induce Th_2 cell immunity to OVA (**Figure 4**), and their elimination never leads to complete abolition of DC activation or subsequent T cell activation (Kool et al., 2008a, 2008b). The most striking finding of our paper is that UA is also released upon primary

exposure of the lung to HDM, as well as upon allergen challenge in already sensitized human asthmatics and mice. Experiments with the UA-degrading enzyme uricase furthermore showed that endogenously released UA promotes Th_2 cell sensitization by amplifying the production of innate pro-Th_2 cytokines that instruct DCs to induce Th_2 cell immunity, like TSLP, GM-CSF, and IL-25 (Barrett and Austen, 2009; Lambrecht and Hammad, 2009), as well as stimulating the activation of inflammatory DCs. Many reported that this innate pro-Th_2 cytokine release and DC activation depends on TLR4 triggering on lung epithelial cells via endotoxin contained in the HDM allergen (Hammad et al., 2009, 2010; Trompette et al., 2009). We now show that TLR4 triggering is functionally connected to the induction of UA production in epithelial cells and macrophages. Most probably this occurs via induction of the enzymatic activity of the rate-limiting uric acid-synthesizing enzyme xanthine oxidoreductase, leading to production of UA inside peroxisomes. Alternatively, increased UA concentration could also result from reduced catabolism from urate oxidase. Although we have not formally investigated the mechanisms of TLR4-driven induction of UA production, it is known that UA is found in the lining fluid of the airway epithelial layer *in vivo* and is induced in response to various types of oxidative stress like ozone exposure or respiratory viral infection (Akaike et al., 1990; Papi et al., 2008). The xanthine oxidoreductase gene is induced as part of an innate immune response to various stimuli like LPS, tissue hypoxia, and early innate cytokines like IL-1, and the promotor region contains an NF-κB control element (Hassoun et al., 1998; Vorbach et al., 2003). How exactly locally released UA affects the innate cytokine production by epithelial cells is at present unclear, nor do we have any indication that UA concentration would be so high that UA crystals are formed in the lung lining fluid. Because of physicochemical constraints, we have been unable to transfer UA crystals to the lungs of mice to directly probe for effects on epithelial cells without mortality of the mice. One possibility that needs further exploration is the fact that the antioxidant properties of UA in the lung lining fluid affect the potential of bronchial epithelial cells to synthesize, correctly fold, and secrete proteins, processes sensitive to oxidative stress (Santos et al., 2009). We have also reported previously that ATP is released in the airways of OVA-challenged sensitized mice, as well as allergen-challenged patients, indicating that a DAMP can contribute to asthma pathogenesis (Idzko et al., 2007). Because ATP is a well-known activator of the NLRP3 inflammasome (Franchi et al., 2007), acting via the $P2X_7$ purinergic receptor that is a potassium efflux channel, it made sense to address whether either alum-induced or HDM-induced asthma relied on $P2X_7$. Experiments in $P2x_7R^{-/-}$ mice did not, however, reveal a crucial role of this receptor in asthma development, again arguing against a predominant role of NLRP3 in asthma. However, there are many ways by which ATP can activate innate and structural cells of the lung to promote asthma and Th_2 cell development, because the purinergic receptor family is very broad and widely expressed among others on eosinophils (Kobayashi et al., 2010). Recently, several groups studying Th_2 cell response induction to the protease allergen papain or to gastrointestinal parasites have reported that DCs are poor inducers of Th_2 cell immunity that are neither sufficient nor required for Th_2 lymphocyte differentiation, basophils being clearly superior (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). However, it was recently also shown that for HDM sensitization, inflammatory DCs are the antigen-presenting cells that provide the first instruction to Th_2 cell development, whereas basophils act to enhance these responses (Hammad et al., 2010). Given the fact that the relative contribution of DCs versus basophils

seems to depend on the type of Th_2 cell stimulus or the route of injection (Tang et al., 2010), it was therefore of interest to study the involvement of DCs in mediating the Th_2 cell adjuvant activities of UA injected into the peritoneal cavity. Employing the logic of Koch's postulates, we first found that inflammatory type DCs and their monocytic precursors were recruited to the site of UA injection, that depletion of $CD11c^{hi}$ dendritic cells abolished UA-driven Th_2 cell responses, and that adoptive transfer of inflammatory DCs to the peritoneal cavity was sufficient to induce Th_2 cell immunity. Moreover, because we did not observe basophil recruitment to the draining lymph nodes of the peritoneal cavity (data not shown), we believe that these experiments are sufficient proof that DCs are the cells driving Th_2 cell immunity to OVA+UA. Recently, UA crystals were shown to directly engage cholesterol- rich cellular membranes of DCs in a receptor-independent manner, leading to activation of Syk and downstream PI3K signaling (Ng et al., 2008). Experiments with *in vitro* and *in vivo* Syk inhibitors, as well as kinase-dead Pik3cdD910A mice, revealed that this pathway was relevant for early *in vivo* recruitment of inflammatory DCs to the site of injection and *in vivo* development of Th_2 cell immunity to OVA+UA. Although these manipulations could also affect other cells, the use of mice specifically lacking Syk expression in DCs allowed us to conclude that at least the DC is a predominant sensor of UA crystals. How exactly DCs exposed to UA subsequently polarize Th_2 cell immune responses is more speculative, but again probably involves Syk signaling. We observed that DCs exposed to OVA+UA produced IL-1β, IL-6, and TNF-α in the absence of the polarizing cytokine IL-12, in a Syk-dependent manner. We and others have previously reported that absence of IL-12 production is a prerequisite for inflammatory DCs to be able to induce Th_2 cell immunity, because retroviral overexpression of IL-12 abolishes DC-driven Th_2 cell development and subsequent asthma (Eisenbarth et al., 2002; Kuipers et al., 2004), whereas others reported that IL-6 and TNF-α are all involved in promoting DC-driven Th_2 cell development in asthma (Dodge et al., 2003; Ebeling et al., 2007; Eisenbarth et al., 2002; Rincon et al., 1997). One report demonstrated that Syk signaling in DCs is crucial for Th_2 cell-dependent asthma development driven by adoptive transfer of DCs, but it is unclear how this relates to polarizing cytokine secretion (Matsubara et al., 2006). Further definitive experiments will involve neutralization of individual cytokines to address these issues in detail. In conclusion, we have identified an unexpected role for UA as a crucial initiator and amplifier of Th_2 cell immunity and allergic inflammation in the mouse by activating inflammatory DCs. At the same time we have identified a pathway of immune activation by UA crystals that is independent of their well-known capacity to trigger the NLRP3 inflammasome. The fact that neutralization of uric acid at the time of allergen challenge reduces the salient features of asthma, and the fact that increased UA concentrations are found in patients after allergen challenge, open up the prospects that this pathway could be used for novel therapeutics in asthma.

Experimental procedures

Human Subjects Twenty-one nonsmoking patients with mild allergic asthma (**Table S1**) were recruited based on airway hyperresponsiveness, positive allergen skin prick tests, elevated total or specific IgE concentrations, and a dual reaction after allergen challenge (Lommatzsch et al., 2006). The calculation of the individual provocation dose was performed based on responsiveness to inhalation allergen challenge (Lommatzsch et al., 2006). Inhaled and segmental allergen challenges were separated by at least 4 weeks. Segmental allergen challenge was performed as described (Lommatzsch et al., 2006). Corticosteroids were withdrawn at least 7 days before challenge. Patients gave

their written informed consent, and he study was approved by the local ethics committee of the University Hospital Rostock, Germany.

Mice BALB/c mice and C57BL/6 mice (6–9 weeks old) were purchased from Harlan (Zeist, The Netherlands). *Nalp3*$^{-/-}$, *Pycard*$^{-/-}$, *Tlr4*$^{-/-}$, *P*$_2$*x*$_7$*R*$^{-/-}$, *Myd88*$^{-/-}$, *Il1r*$^{-/-}$, and WT C57BL/6 mice (6–10 weeks old) were kindly provided by J. Tschopp (University of Lausanne, Swizterland) and B. Ryffel (University of Orleans, France). Pik3cdD910A were kindly provided by B. Vanhaesebrouck (Queen Mary University London). To generate mice lacking Syk specifically in DCs, *Cd11c*-Cre mice were crossed to Sykb $^{fl/fl}$ mice (unpublished observations). Bone marrow of these mice was used to generate chimeric mice, by sublethally irradiating C57BL/6 mice (5Gy), followed 4 hr later by intravenous (i.v.) injection of 2 x 10^6 BM cells. Chimeric mice were used 10 weeks after hematopoietic reconstitution. All experiments were approved by the animal ethics committee at Ghent University and Erasmus University Medical Center.

Monosodium Urate Crystal Preparation MSU crystals were prepared as described (Chen et al., 2006). In brief, saturated uric acid (5 mg/mL, Sigma) in 0.1M borate buffer (pH 8.5) was incubated at room temperature for 48 hr. After harvesting the crystals, they were washed with alcohol and acetone and air-dried for at least 2 days. Crystals were between 5 and 25 μm long and contained <0.005 EU/mL endotoxin.

Treatment with Uricase For intraperitoneal injections, mice received 50 U of uricase (Sigma-Aldrich) or rasburicase (Sanofi-Synthelabo) in 100 ml PBS 5 min prior to injection of OVA+alum. For intrapulmonary administration, HDM was mixed with 10 U uricase and injected intranasally into 40 ml.

Induction of Allergic Airway Inflammation To test the adjuvant properties of MSU crystals, mice were injected intraperitoneally (i.p.) with 10 μg of OVA (Worthington) mixed with increasing amounts of MSU crystals (1–7.5 mg in 500 ml). Mice were boosted i.p. on day 7 with OVA and challenged with OVA aerosols (Sigma, 1% for 30 min) on days 17–19. On day 20, mice were analyzed for the presence of Th_2 cell-dependent eosinophilic airway inflammation. To induce HDM-driven eosinophilic airway inflammation, anesthetized mice were administered intranasaly (i.n.) with 40 μl of house dust mite extracts (100 μg, Greer Laboratories) on day 0 and challenged on days 7–11 with HDM. On day 14, mice were sacrificed and different parameters were analyzed. Assessment of bronchial hyperreactivity and histological analysis of goblet cell hyperplasia was measured as described previously (Hammad et al., 2009).

Immune Analysis To determine the degree of airway eosinophilia, bronchoalveolar lavages (BAL) were performed by injecting 1 ml of PBS containing 0.01mM EDTA. Cells were stained with FITC anti-I-Ad and I-E^d, PE-labeled anti-SiglecF, PerCp-labeled anti-CD3 and anti-CD19, and APC-labeled anti-CD11c (all from BD Biosciences). Peritoneal inflammatory cells were stained as described (Kool et al.,2008b). Data were collected on a FACS Aria II (Becton Dickinson) and were analyzed with FlowJo software (Treestar, Inc.). The concentration of UA (Amplex Red Uric Acid; Uricase assay, Invitrogen), GM-CSF, IL-33, TSLP, and IL-25 (all R&D Systems) was measured in BAL fluid and lung homogenates by ELISA 2 and 24 hr after HDM administration. The concentration of Th_2 cytokines in BAL fluids was measured 24 and 72 hr after the last challenge, respectively. OVA-specific IgG_1, IgE, and IgG_{2a} were measured in sera of mice sensitized and challenged with OVA by ELISA (antibodies from R&D Systems).

Uric Acid Staining in the Lung Two hours after the i.n. administration of PBS or HDM, lungs were inflated with PBS and OCT and frozen at -80°C. Ten mm thick sections were fixed with 4% formaline for 10 min and stained with rabbit anti-uric acid antibodies (Abcam) followed by Cy3-labeled goat anti-rabbit antibodies (Jackson Laboratories), blocking with rabbit serum, biotin-labeled anti-peroxisomal membrane protein 70 (Genetex), and FITC-labeled anti-MHCII (BD Bioscience), followed by streptavidin Alexa Fluor 647. Nuclei were counterstained with 40,60 diamidino-2-phenylindole (DAPI). Images were collected on a Zeiss LSM 710 equipped with a Ar 488 nm laser, a He-Ne 561 nm laser, a He-Ne 633 nm laser, and a Mai Tai femtosecond-pulsed laser tuned at 800 nm to excite DAPI. Images were analyzed with Imaris (Bitplane).

qPCR on Xanthine Oxido Reductase RNA was isolated from BAL cells with High Pure RNA Isolation Kit (Roche) with on-column DNase I treatment. 100 ng RNA was reverse transcribed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Quantitative PCR was performed with LightCycler 480 Probes Master (Roche) and primers (xanthine oxidoreductase; forward, AGGGATTCCGGACCTTTG; reverse, GCAGCAGTTTGGGTTGTTTC) and probe mixes (probe 69; Roche). PCR conditions were 10 min at 95°C, followed by 45 cycles of 10 s at 95°C and 50°C for 1 min with a LightCycler 480 Instrument (Roche). PCR amplification of the housekeeping gene hypoxanthine-guanine phosphoribosyl-transferase (HPRT) was performed during each run for each sample to allow normalization between samples.

Statistical Analysis For all experiments unless stated otherwise, the difference between groups was calculated with the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Comparison of the human BAL fluid UA concentration was performed with the Wilcoxon-signed rank test. Correlations between

the human data were tested with Pearson's correlation test. Differences were considered significant when $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.005$ (***).

Supplemental information

Supplemental Information includes six figures and one table and can be found within this thesis and online at doi:10.1016/j.immuni.2011.03.015.

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Recent findings

A study on nasal secretion of chronic rhinosinusitis patients showed that uric acid was significantly increased after provocation and remained elevated during the observation period (Rank et al., 2013). If ozone was used to induce inflammation in asthmatics, no difference was seen on uric acid release (Esther et al., 2011). We found UA release to be dependent on TLR4 signalling. The finding of Esther also points to a specific release of UA upon allergen exposure, rather than UA as an danger signal.

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Supplemental information

	Sex	Age (y)	FEV1 (% pred)	Total IgE (kU l^{-1})	Specific IgE (kU l^{-1})	Asthma duration (y)	Allergen	Allergen dose (AU)
1	f	23	114	246	5.5	17	Birch	110
2	f	20	112	2047	27.5	14	Birch	53
3	m	23	95	345	11.8	5	Birch	400
4	m	23	77	548	26.1	5	Birch	76
5	f	25	105	64	16.9	2	HDM	1.8
6	f	24	102	155	5.21	21	Rye	4.25
7	m	20	114	141	18.7	13	Rye	1150
8	f	28	105	22	6.85	10	Birch	64
9	m	34	114	172	30.7	10	Rye	46
10	m	24	94	368	66.3	17	HDM	49
11	m	24	105	104	8.3	4	HDM	71
12	f	22	110	134	39.6	2	Rye	340
13	m	24	97	1335	>100	16	Birch	123
14	m	23	107	622	46.8	4	HDM	2180
15	f	30	100	298	45.1	12	HDM	51
16	m	31	120	129	22.4	6	Birch	1450
17	m	27	83	430	44.8	20	HDM	5.3
18	m	39	97	770	29.7	26	HDM	2.86
19	m	25	112	57	14.7	13	HDM	1340
20	m	25	80	226	37.0	4	Birch	0.056
21	f	20	98	504	66.9	16	HDM	1460

Table S1: Clinical characteristics of asthmatic patients The table displays sex and age (in years) of the patients, the pre-bronchodilator forced expiratory volume in the first second (FEV1, % predicted), the serum levels of total (normal range: < 100 kU l-1) and allergen-specific (normal range: < 0.7 kU l-1) Immunoglobulin E (IgE) levels, the asthma duration (in years), the allergen, and the dose (in allergen units, AU) used for segmental allergen challenge. Median age of the patients was 25 years old, range 20-31.

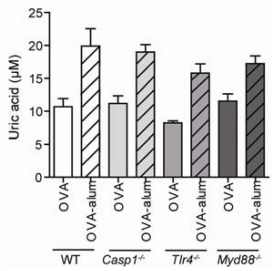


Figure S1: OVA-alum induces UA independent of the TLR/IL-1R axis. *Casp1-/-*, *Tlr4-/-*, *Myd88-/-* mice and their control littermates were injected with OVA-alum or OVA alone. 2hrs later the peritoneal lavage was taken and UA levels were determined.

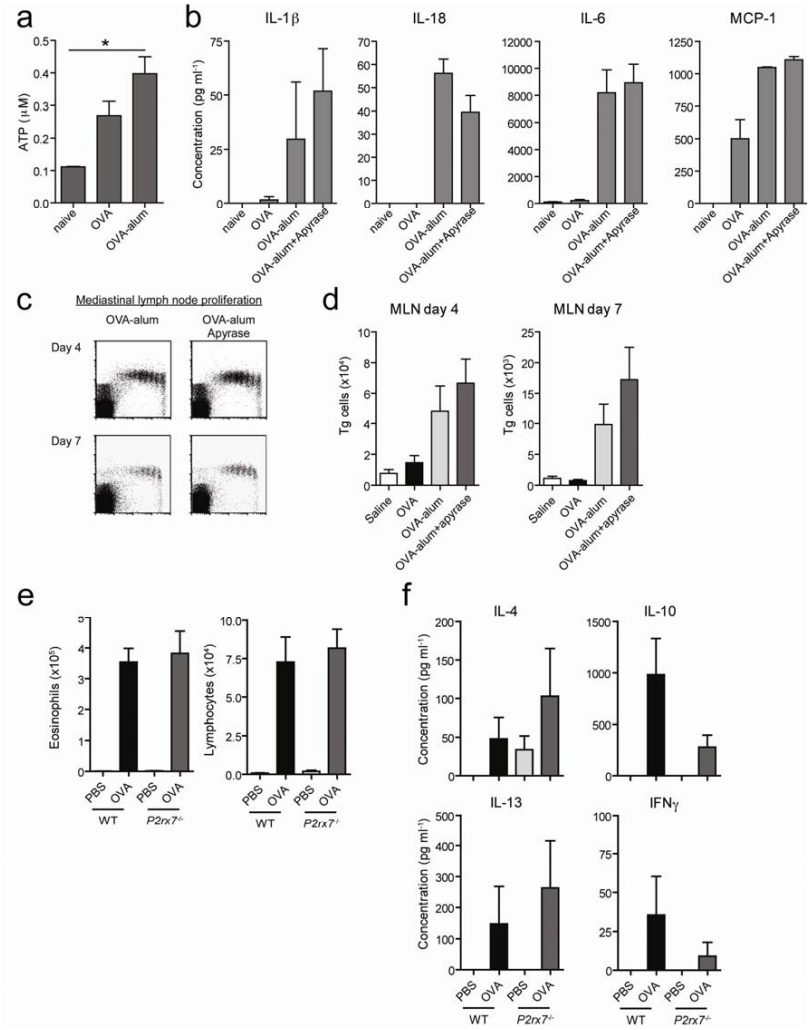


Figure S2: OVA-alum responses are independent of ATP or P2X$_7$. (a) BALB/c mice were injected i.p. with OVA or OVA-alum and 2 hrs the levels of ATP were determined in the peritoneal lavage. (b) The peritoneal lavage was also examined for the levels of IL-1β, IL-6, IL-18, and MCP-1 by ELISA. (c) 2.5*10^6 CFSE-labeled DO11.10 OVATCR Tg CD4$^+$ T cells were given to BALB/c mice one day prior to OVA-alum. Mice were treated 5 min before OVA-alum with apyrase. Four and 7 days later the mediastinal lymph node cells were collected and examined by flowcytometry for divisions. (d) Total numbers of OVA-TCR Tg cells are depicted. (e) WT or *P2rx7*-/- mice were sensitized on day 0 with alum (PBS) or OVA-alum (OVA), boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17-19. Mice were sacrificed on day 20, and the cellular composition in the BALf is shown. (f) On day 20 the mediastinal lymphnode cells were restimulated with OVA (10 μg/ml) for four days. Cytokines were determined in the supernatant by ELISA. Data are shown as mean ± SEM, * p<0.05, n=4-6 mice/group.

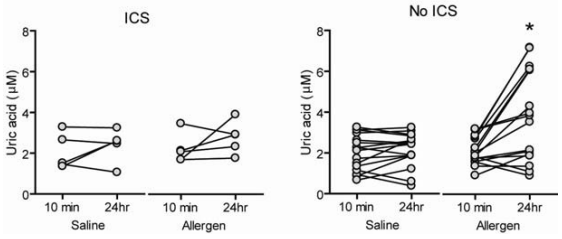


Figure S3: Uric acid levels are only elevated in patients not receiving inhaled corticosteroids. Uric acid levels in BALf of asthmatic patients 10 min or 24h after segmental bronchial allergen or saline challenge, defined as patients on ICS (inhaled corticosteroids) prior to segmental challenge or patients not on ICS.

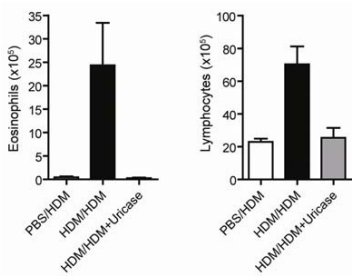


Figure S4: Uricase treatment during challenge in a milder HDM model. Mice were sensitized i.n. on day 0 with PBS or 1 µg HDM. During challenge (day 7-11) with 10 µg HDM mice were treated with uricase. On day 14, BALf was examined for the presence of eosinophils and lymphocytes.

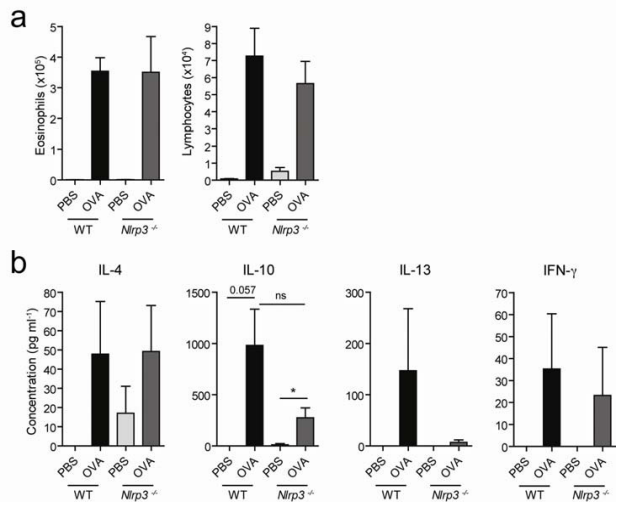


Figure S5: OVA-alum driven Th_2 development in a model of asthma develops normally in *Nlrp3-/-* mice. (**a**) WT C57BL/6 or Nlrp3-/- mice were immunized with OVA-alum i.p. on day 0. On day 7, mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17-19. On day 20, BALf was analyzed for the presence of eosinophils and lymphocytes. (**b**) MLN cytokine production was measured as in Supplementary Fig. 2. Data are shown as mean ± SEM, * p<0.05, ** p<0.01, n=4-6 mice/group.

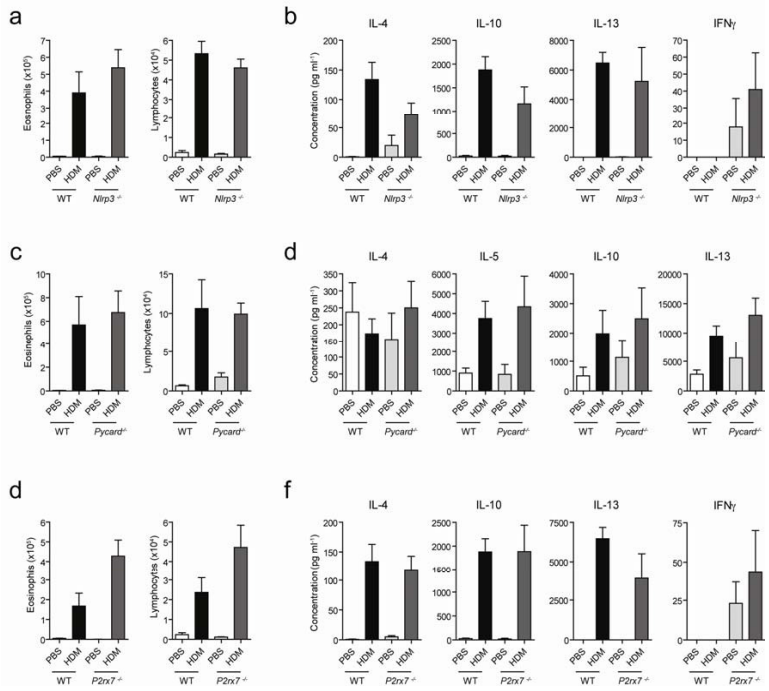
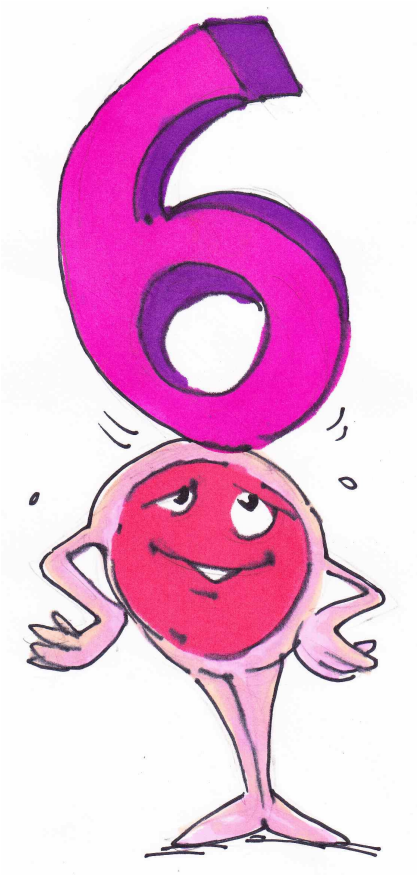


Figure S6: HDM driven asthma develops normally in *Nlrp3-/-* , *Pycard-/-* , and *P2rx7-/-* mice WT C57BL/6 or *Nlrp3*-/- (a, b), *Pycard*-/- (c,d) or *P2rx7*-/- (e,f) mice were sensitized i.n on day 0 with PBS or HDM, and challenged i.n with HDM on days 7-11. On day 14, BALf was examined for the presence of eosinophils and lymphocytes (a, c, e). MLN cytokine production was measured as in Supplementary Fig. 2 (b,d,f).



Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33.

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Various reports in the past studied the role of IL-1 in asthma, however none of these were done with protease containing allergens such as house dust mite. Here we showed for the first time the cytokine cascade following antigen exposure in the lung. This paper reveals an important role for epithelial cells and IL-1α in the sensitization to house dust mite.

Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33

Abstract

House dust mite (HDM) is one of the most common allergens worldwide. In this study, we have addressed the involvement of IL-1 in the interaction between HDM and the innate immune response driven by lung epithelial cells (ECs) and dendritic cells (DCs) that leads to asthma. Mice lacking IL-1R on radioresistant cells, but not hematopoietic cells, failed to mount a Th_2 immune response and did not develop asthma to HDM. Experiments performed *in vivo* and in isolated air–liquid interface cultures of bronchial ECs showed that TLR4 signals induced the release of IL-1α, which then acted in an autocrine manner to trigger the release of DC-attracting chemokines, GM-CSF, and IL-33. Consequently, allergic sensitization to HDM was abolished *in vivo* when IL-1 α, GM-CSF, or IL-33 was neutralized. Thymic stromal lymphopoietin (TSLP) became important only when high doses of allergen were administered. These findings put IL-1α upstream in the cytokine cascade leading to epithelial and DC activation in response to inhaled HDM allergen.

Introduction

Allergic asthma is characterized by a Th_2-dominated immune response to inhaled allergens like house dust mite (HDM), cockroach, and animal dander, which leads to eosinophilic inflammation of the airways, goblet cell metaplasia (GCM), and bronchial hyperreactivity (Barnes, 2008). The incidence of allergic sensitization to inhaled allergens has steadily risen over the last 60 yr, and in some countries up to one third of children have a positive skin prick test to environmental allergen (Umetsu et al.,2002). The various molecularly defined allergens found within the HDM *Dermatophagoides pteronyssinus* are the most common triggers of allergic asthma worldwide, which has led to the development of animal models that use inhaled HDM extracts to study the cellular and molecular mechanisms of allergic sensitization. Recent studies have shown that inhaled HDM extract stimulates Th_2 immunity by acting on barrier epithelial cells (ECs), antigen-presenting DCs, and innate immune cells like basophils and mast cells (Hammad et al., 2010; Wills-Karp, 2010; Lambrecht and Hammad, 2012). One predominant way by which HDM induces Th_2 immunity is by triggering the TLR4 receptor expressed on bronchial ECs, through its major allergen Der p 2 together with endotoxin contained in fecal pellets of the mite (Trompette et al., 2009). There is also evidence that components of HDM trigger C-type lectin or protease-activated receptors and thus contribute to the recruitment and activation of innate and adaptive immune cells (Barrett et al., 2009; Nathan et al., 2009; Lewkowich et al., 2011). Research from various laboratories has shown that triggering of pattern recognition receptors on ECs leads to release of innate pro-Th_2 cytokines like thymic stromal lymphopoietin (TSLP), GM-CSF, IL-25, and the IL-1 family member IL-33 that share the capacity of activating DCs (Hammad et al., 2009; Phipps et al., 2009; Kool et al., 2011). In this regard, TSLP has received much attention as it promotes Th_2 development by DCs, activation of innate lymphoid cells, and basophil hematopoiesis (Kitajima et al., 2011;

Siracusa et al., 2011). Transgenic (Tg) overexpression of TSLP in lung ECs led to enhanced Th_2 responses to inhaled environmental antigens (Headley et al., 2009; Lei et al., 2011). Studies in human severe asthmatics demonstrated increased levels of TSLP messenger RNA and protein in bronchial biopsy specimens and identified associations between TSLP promoter region polymorphisms and risk of developing asthma (Harada et al., 2011; Shikotra et al., 2012). Although TSLP has become a prime target for intervention in allergic disease, other cytokines like IL-33, GM-CSF, or IL-25 could also control Th_2 immunity to inhaled allergens (Fort et al., 2001; Hurst et al., 2002; Cates et al., 2004; Besnard et al., 2011). The signals that control innate cytokine and chemokine release from bronchial ECs are poorly understood, and it is particularly unclear whether released cytokines could act as a cascade, with one cytokine stimulating the release of another, and thus acting as a controller of allergic sensitization. This possibility was raised when *in vitro* studies on cultured lung ECs found that IL-1 is a potent upstream inducer of TSLP production (Allakhverdi et al., 2007; Lee and Ziegler, 2007; Lee et al., 2008). In this study, we have addressed the involvement of IL-1 in the interaction between HDM allergen, lung ECs, and DCs that leads to asthma. We found that mice lacking IL-1R on radioresistant cells, but not hematopoietic cells, were totally protected from mounting Th_2 immunity and asthma to low doses of HDM. Experiments performed *in vivo* and in isolated air–liquid interface (ALI) cultures of bronchial ECs showed that this was caused by the TLR4-mediated release of IL-1α, which acted in an autocrine manner on bronchial ECs to release DC-attractive chemokines, GM-CSF, and IL-33 but not TSLP. Consequently, allergic sensitization to low-dose HDM was only abolished *in vivo* when IL-1α, GM-CSF, or IL-33 was neutralized. However, TSLP and its receptor became important when mice were exposed to high doses of HDM. These findings put IL-1α upstream in the cytokine cascade leading to epithelial and DC activation in response to inhaled HDM allergen.

Results

IL-1RI signaling is crucial for development of asthma to HDM

IL-1α and IL-1β are closely related cytokines that share biological activity by acting exclusively on the IL-1R1 on various cell types. To address how these two cytokines affect development of asthma, we sensitized WT and *Il1r1*$^{-/-}$ mice using 1 µg HDM or PBS as a control and challenged all mice intranasally 1 wk later on five consecutive days with 10 µg HDM. This protocol differs from previously published work on HDM-driven asthma, where we (Hammad et al., 2009) and others (Cates et al., 2004; Lewkowich et al., 2005; Phipps et al., 2009) administered higher doses of HDM. Using this protocol, we observed that HDM-sensitized WT mice developed signs of allergic asthma, such as influx of eosinophils and lymphocytes in the bronchoalveolar lavage (BAL) fluid (**Fig. 1 A**), peribronchial and perivascular infiltration of mononuclear cells, lymphocytes, and eosinophils, and GCM (**Fig. 1 B**), whereas sham PBS-sensitized mice exposed to HDM did not. In their serum, HDM-sensitized mice produced HDM-specific IgG_1 and IgE, whereas Th_1 cytokine–dependent IgG_{2a} antibodies were not induced (**Fig. 1 C**). HDM-sensitized and challenged mice developed bronchial hyperreactivity to methacholine, whereas sham-sensitized mice did not (**Fig. 1 D**). These features of asthma were accompanied by enhanced production of Th_2 cytokines IL-13, IL-5, and IL-10 in the mediastinal LNs (MLNs; **Fig. 1 E**). The levels of IL-17 and IFN-γ were not induced significantly by active HDM sensitization. Importantly, when these experiments were performed in *Il1r1*$^{-/-}$ mice, there was a strong

reduction in lung eosinophilia and lymphocytosis, GCM, HDM-specific IgG_1 and IgE production, and bronchial hyperreactivity to methacholine. In addition to severely impaired Th_2 cytokine production, we also noticed that production of IL-17 was impaired in *Il1r1*$^{-/-}$ mice, consistent with a known role for this cytokine in Th_{17} development (Sutton et al., 2006).

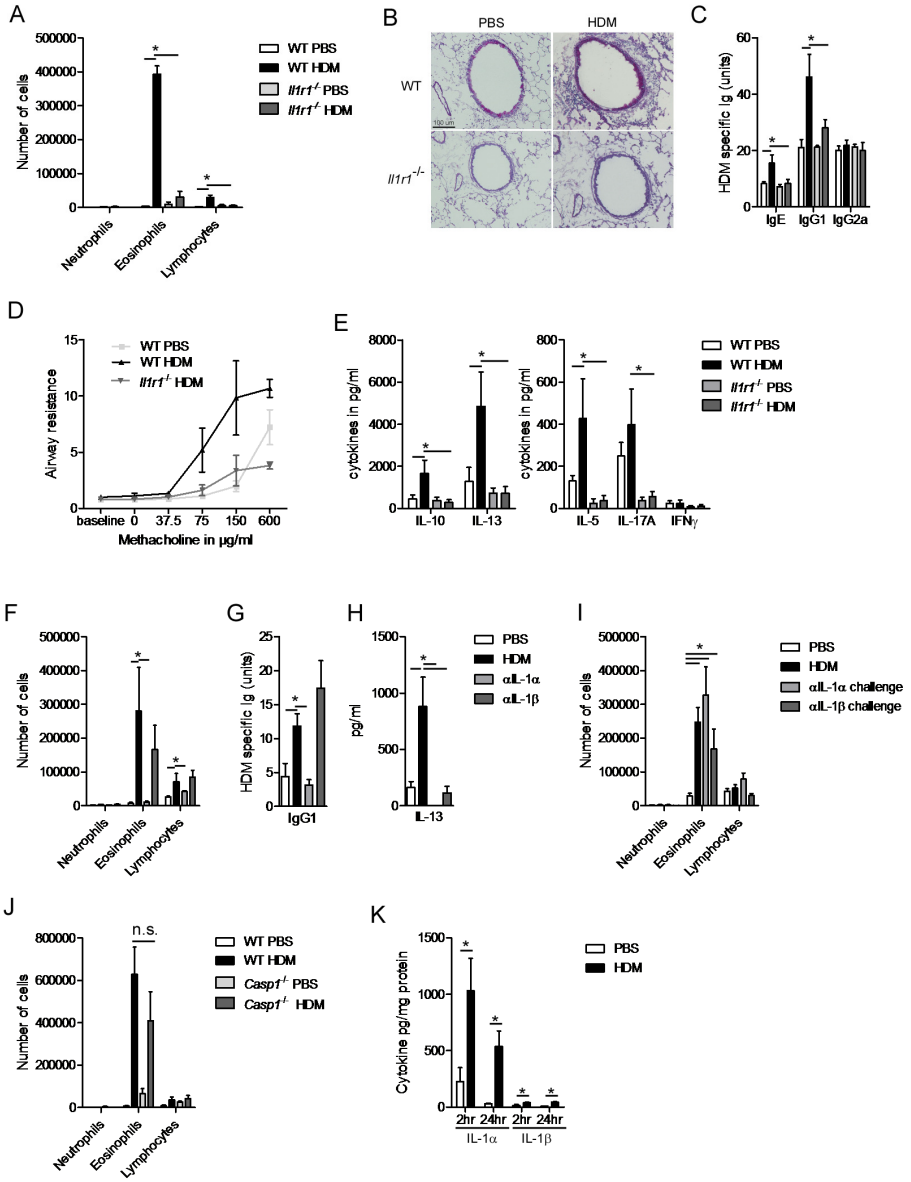

Figure 1. IL-1RI signaling is crucial for the development of HDM-induced asthma. (A–E) WT and *Il1r1*$^{-/-}$ mice were sensitized on day 0 with HDM or PBS and were challenged with HDM on days 7–11. (**A**) Differential cell counts were determined by flow cytometry 72 h later. (**B**) PAS staining of lung sections. (**C**) Levels of serum HDM–specific Igs. (**D**) Airway resistance in response to increasing concentrations of methacholine. (**E**) Cytokine levels in MLN cells restimulated for 3 d with 15 µg/ml HDM. (F–H) C57BL/6 mice were sensitized with HDM in the presence or absence of blocking antibodies to IL-1α or IL-1β and were challenged with HDM. (**F**) Differential cell counts were determined by flow cytometry 72 h later. (**G**) IgG1 levels in sera. (**H**) IL-13 levels in MLN cells restimulated for 3 d with HDM. (**I and J**) C57BL/6 mice were sensitized with HDM or PBS and were administered blocking antibodies to IL-1α or IL-1β on the last 3 d of HDM challenge. (**I**) Differential cell counts were determined by flow cytometry 72 h later. (**J**) WT and *Casp1*$^{-/-}$ mice were sensitized with HDM or PBS as a control and were re-challenged with HDM. Differential cell counts were determined by flow cytometry 72 h later. (**K**) WT mice were administered with PBS or HDM. IL-1α and IL-1β contents were determined in lung homogenates 2 and 24 h later. *, $P < 0.05$. Results show one representative experiment out of three. Five to six mice/group were used. Results are shown as mean ± SEM.

HDM sensitization depends mainly on IL-1α, not IL-1β.

These experiments demonstrated that IL-1RI signaling is crucial for development of asthma but did not address whether IL-1 is required during sensitization (i.e., the first administration of HDM) or challenge phase (days 7–12) of the response. Also, as IL-1α and IL-1β both trigger the IL-1RI equally well, we addressed their relative contribution during sensitization or challenge. Neutralizing IL-1α with a blocking antibody during sensitization led to strongly reduced airway eosinophilia and lymphocytosis (**Fig. 1 F**), serum HDM–specific Ig levels (**Fig. 1 G**), Th_2 cytokines (**Fig. 1 H**; only IL-13 shown as representative Th_2 cytokine), and periodic acid-Schiff (PAS)–positive GCM (not depicted). Neutralizing IL-1β during sensitization had no statistically significant effect on eosinophilic influx in the lung and on serum HDM–specific Igs (**Fig. 1, F and G**). IL-1β neutralization did reduce levels of Th_2 cytokine production in the MLNs, however to a lesser extent than IL-1α blockade (**Fig. 1 H**). When either IL-1α or IL-1β was blocked during the challenge phase, there was no effect on BAL fluid cellular composition (**Fig. 1 I**) or any of the other parameters (not depicted). Caspase-1 (a.k.a. IL-1 converting enzyme 1) is necessary to process pro–IL-1β into biologically active secreted IL-1β, whereas it is not necessary for IL-1α biological activity (Dinarello, 2009). We therefore sensitized WT and *casp1*$^{-/-}$ mice to HDM and challenged them with HDM. No differences on lung inflammation (**Fig. 1 J**) or any of the other parameters (not depicted) were observed in *casp1*$^{-/-}$ mice, further supporting the idea that mainly IL-1α is necessary for HDM-induced Th_2 immunity. One possible explanation for the differential effect of IL-1α versus IL-1β neutralization on development of HDM asthma could be different levels of production of either cytokine. We therefore measured the levels of IL-1α and IL-1β in lungs, 2 and 24 h after HDM administration of naive mice and found that IL-1α levels were strongly induced at 2 and 24 h, whereas IL-1β was barely detected (**Fig. 1 K**).

IL-1RI is necessary on radioresistant cells of the lungs

Because we observed that IL-1α was necessary for promoting Th_2 immunity to natural allergens in the lung, we next questioned how it promoted Th_2 immunity. The IL-1R1 is not only expressed on various immune cells like macrophages, DCs, T lymphocytes, mast cells, and eosinophils, but also on structural cells like fibroblasts and ECs. To address on which cell type IL-1R was necessary, we made a series of bone marrow chimeric mice. WT and *Il1r1*$^{-/-}$ mice were sublethally irradiated and received donor WT or *Il1r1*$^{-/-}$ bone marrow to allow for hematopoietic reconstitution. 10 wk later, we sensitized and challenged them with HDM. As in previous experiments (**Fig. 1**), WT → WT chimeric mice mounted higher degrees of airway eosinophilia and lymphocytosis (**Fig. 2 A**), serum HDM–specific IgG_1 (**Fig. 2 B**), and GCM (**Fig. 2 C**) compared with *Il1r1*$^{-/-}$ → *Il1r1*$^{-/-}$ mice, essentially illustrating that the irradiation and chimerism procedure did not affect the fundamental outcome of the experiment previously performed in unmanipulated WT and *Il1r1*$^{-/-}$ mice. We observed a similar reduction in BAL fluid eosinophilia and lymphocytosis, HDM-specific Igs, and GCM in mice that lacked the IL-1RI on radioresistant structural cells (WT → *Il1r1*$^{-/-}$), whereas no reduction was seen in mice lacking IL-1RI on hematopoietic cells (*Il1r1*$^{-/-}$ → WT).

Autocrine IL-1α acts on ECs to promote production of chemokines and innate pro-Th2 cytokines

Because of their exposed position as barrier cells of the lungs, bronchial ECs are prime candidates among radioresistant cells for producing IL-1, as well as responding to it. To address this possibility, we set up ALI cultures of primary ECs obtained from digested tracheas of various WT and gene- deficient mice. When these cultures were stimulated with HDM extract, they produced mainly IL-1α but not IL-1β (**Fig. 2 D**), reflecting the situation seen upon *in vivo* exposure of the lung to HDM (**Fig. 1 K**). We and others have previously reported that TLR4 stimulation of ECs is a crucial event in the development of Th_2 immunity to HDM (Hammad et al., 2009; Trompette et al., 2009). When ALI cultures were set up using tracheal ECs of *Tlr4*$^{-/-}$ mice, HDM was less able to induce IL-1α production (**Fig. 2 E**). When ALI cultures were set up derived from *Il1r1*$^{-/-}$ mice, however, HDM was however fully able to induce IL-1α production (**Fig. 2 E**). Moreover, *in vivo*, the levels of IL-1α were also increased in the BAL fluids of WT mice administered with HDM (**Fig. 2 F**). The HDM-induced production of IL-1α seen in the BAL of WT animals was absent in *Tlr4*$^{-/-}$ mice (**Fig. 2 F**). In addition, we also found no increase in IL-1α levels in the BAL of mice treated with uricase at the time of HDM administration (**Fig. 2 F**), indicating that uric acid (UA) produced in response to HDM exposure could induce IL-1α production. However, apyrase pretreatment to degrade any HDM-induced ATP production did not show an effect on IL-1α release (**Fig. 2 F**). Various pro-Th_2 innate cytokines like TSLP, GM-CSF, IL-33, and IL-25, as well as chemokines like KC and CCL20 that can be produced by lung ECs, have been found to be induced in the lungs of HDM-exposed mice *in vivo* (Hammad and Lambrecht, 2008). We next questioned whether epithelial IL-1α and IL-1RI signaling was involved in an autocrine loop influencing the production of these factors. When tracheal epithelial ALI cultures of WT mice were stimulated with HDM extracts, GM-CSF and KC were produced, in a process requiring TLR4 (**Fig. 2 G**). The levels of TSLP, IL-25, and IL-33 in ALI cultures were around the detection limit of the cytokine ELISA and were inconsistent between repeat experiments, so we focused our attention on the production of GM-CSF and chemokines. ALI cultures stimulated with rIL-1α produced GM-CSF, CCL20, and KC, and these effects were not seen in *Il1r1*$^{-/-}$ mice, showing that these effects were not caused by some contaminant in the IL-1 preparations (**Fig. 2 G**). Strikingly, HDM was unable to induce GM-CSF and CCL20 production in ALI cultures generated from *Il1r1*$^{-/-}$ mice, essentially demonstrating that autocrine release of IL-1α was inducing these factors in a process requiring TLR4 triggering by HDM. We next studied the *in vivo* production of early innate pro-Th_2 cytokines and chemokines in WT and *Il1r1*$^{-/-}$ mice by measuring their presence in lung homogenates 12 h after administration of HDM intratracheally (i.t.). As shown in **Fig. 2 H**, HDM administration in WT mice led to the production of GM-CSF, IL-33, CCL20, and KC, whereas TSLP and IL-25 were around the limit of detection in this assay (not depicted). The production of GM-CSF, IL-33, and KC but not that of CCL20 was severely reduced in *Il1r1*$^{-/-}$ mice (**Fig. 2 H**), suggesting that these cytokines are downstream of IL-1. Moreover, the blockade of IL-33 at the time of HDM administration did not affect IL-1α production in the lung, showing that IL-1 is upstream of IL-33 (**Fig. 2 I**).

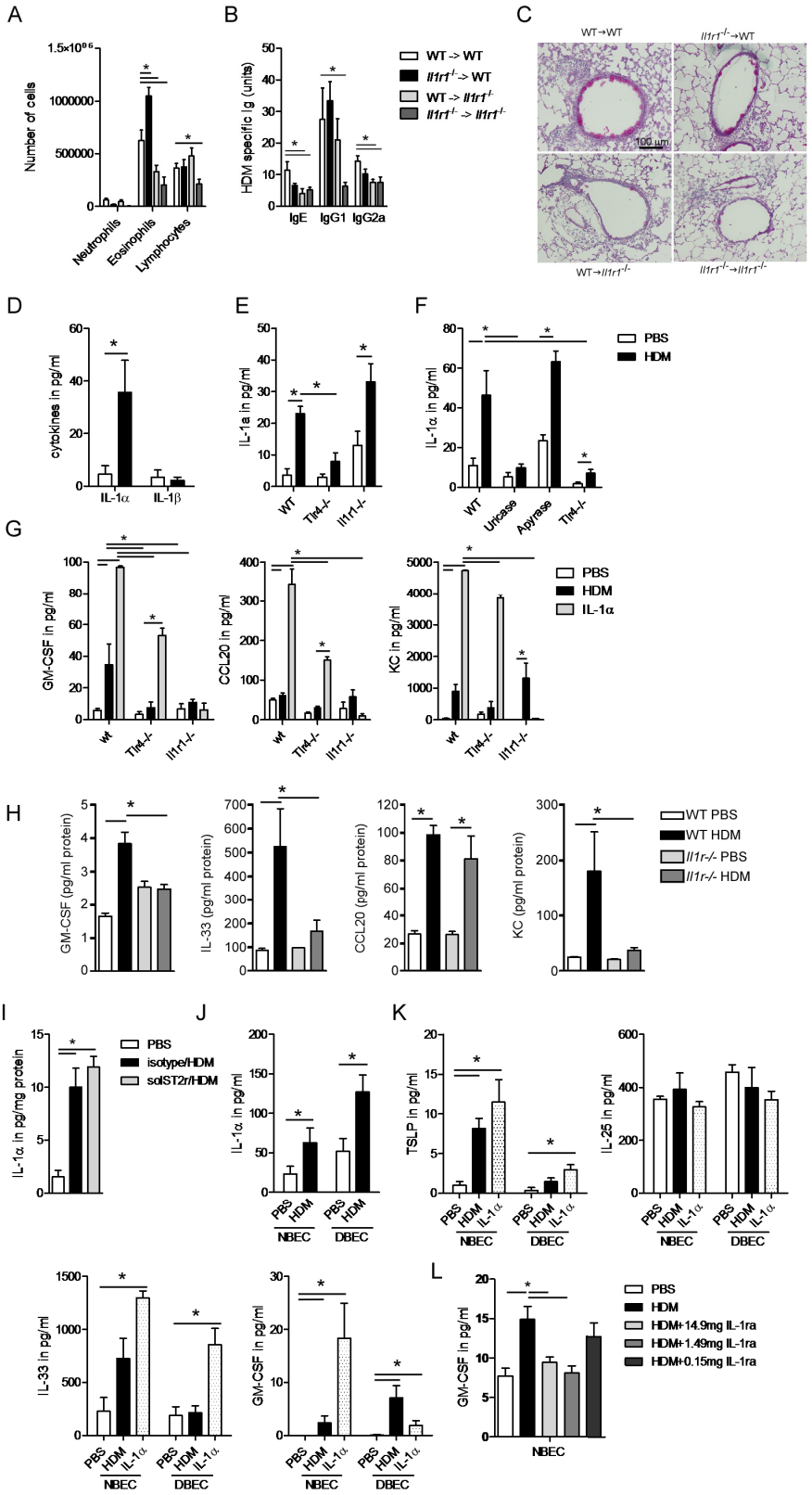

Figure 2. IL-1RI is necessary on radioresistant structural cells of the lungs. (A–C) Various chimeric mice (coded as bone marrow donor → recipient) were sensitized and challenged with HDM as described in Fig. 1. (**A**) Differential cell counts were determined 72 h later. (**B**) Levels of serum HDM–specific Igs. (**C**) PAS staining of lung sections. Five

to six mice/group were used. (**D**) ALI cultures of primary tracheal ECs from WT mice were exposed to HDM or PBS as a control. Levels of IL-1α and IL-1β were measured in supernatants. (**E**) ALI cultures of primary tracheal ECs from WT, *Tlr4*$^{-/-}$, and *Il1r1*$^{-/-}$ mice were exposed to HDM or PBS. Levels of IL-1α were measured in supernatants. (**F**) C57BL/6 WT mice were injected i.t. with PBS, uricase, or apyrase 30 min before exposure to PBS or HDM. *Tlr4*$^{-/-}$ mice were exposed i.t. to PBS or HDM. BAL was collected after 2 h and was analyzed for the presence of IL-1α. (**G**) Levels of GM-CSF, CCL20, and KC were measured in BAL fluids 24 h after exposure to HDM, IL-1α, or PBS. (**H**) GM-CSF, IL-33, CCL20, and KC levels in supernatants of ALI cultures of WT and *Il1r*$^{-/-}$ mice exposed to PBS or HDM. (**I**) WT mice were injected i.p. with solST2r and with HDM i.t. IL-1α levels were measured in lung homogenates 24 h later. (**J**) Primary ECs from healthy donors (NBECs) and from asthmatic patients (DBECs) were exposed to HDM or PBS. Levels of IL-1α were measured 24 h later. (**K**) TSLP, IL-25, GM-CSF, and IL-33 levels in supernatants of NBEC and DBEC cultures exposed to HDM, IL-1α, or PBS. (**L**) Primary ALI cultures of human healthy donors (NBECs) were exposed to PBS or HDM in the presence or not of different doses of IL-1ra. GM-CSF was analyzed in the supernatant of these cultures after 24 h. *, $P < 0.05$. Results show one representative experiment out of three. Results are shown as mean ± SEM.

Human ALI cultures produce IL-1 and GM-CSF in response to HDM allergen

To investigate the translational aspect of these findings, we stimulated ALI cultures of commercially available primary human bronchial ECs (healthy: normal bronchial ECs [NBECs]; asthmatic: diseased bronchial ECs [DBECs]) with HDM allergen after three passages of culture. Exposure of these cultures led to increased IL-1α secretion on the apical side, particularly in ALI cultures set up from DBECs (**Fig. 2 J**). Exposure of the human ECs to HDM also led to GM-CSF and TSLP secretion on the basolateral side but not of IL-25 (**Fig. 2 K**). Exposure of human ALI cultures to IL-1α also led to GM-CSF and IL-33 induction in NBEC cultures, but the GM-CSF response was significantly blunted in DBECs (**Fig. 2 K**). We next addressed whether human IL-1RI signaling was also involved in an autocrine loop influencing the production GM-CSF, as shown previously in mice (**Fig. 2 G**). NBEC cultures were exposed to PBS or HDM in the presence of different doses of a blocking IL-1R antagonist. IL-1Ra did not induce any cell death in the cultures as assessed by the absence of tight junction disruption (not depicted). As shown in **Fig. 2 L**, the addition of IL-1Ra to the cultures decreased the production of GM-CSF induced by HDM, demonstrating that the autocrine release of IL-1α was inducing GM-CSF production in human ECs.

Development of Th_2 immunity to HDM depends on GM-CSF and IL-33

GM-CSF, IL-33, and TSLP are potentially important cytokines that were produced in the epithelial ALI cultures and/or *in vivo* upon HDM exposure in an IL-1R1–dependent manner. To fully understand the importance of these cytokines in the process of sensitization to HDM, we first sensitized and challenged TSLP receptor–deficient (*Crlf2*$^{-/-}$) and WT C57BL/6 control mice to HDM. Using a low dose of HDM to sensitize and challenge mice (1 μg for sensitization followed by 10 μg for challenge for 5 d, as in all experiments), we observed that TSLP receptor signaling was not necessary to induce sensitization to HDM because these knockout mice developed identical eosinophilic and lymphocytic influx in the lungs (**Fig. 3 A**) and identical HDM-specific IgG_1 levels (**Fig. 3 B**) after HDM challenge and even increased cytokine levels after restimulation of MLN cells (**Fig. 3 C**). The levels of IL-17 were increased in *Crlf2*$^{-/-}$ mice. In addition, the administration of a low dose of HDM did not induce TSLP production in the BAL fluids compared with mice exposed to PBS (**Fig. 3 D**). Similar results were obtained when *Tslp*$^{-/-}$ C57BL/6 mice or BALB/c *Crlf2*$^{-/-}$ were used for experiments, suggesting that these results hold true across two strains of mice and when either receptor or its ligand is inhibited (not depicted). However, when we used a higher dose of HDM (100 μg) to sensitize and challenge

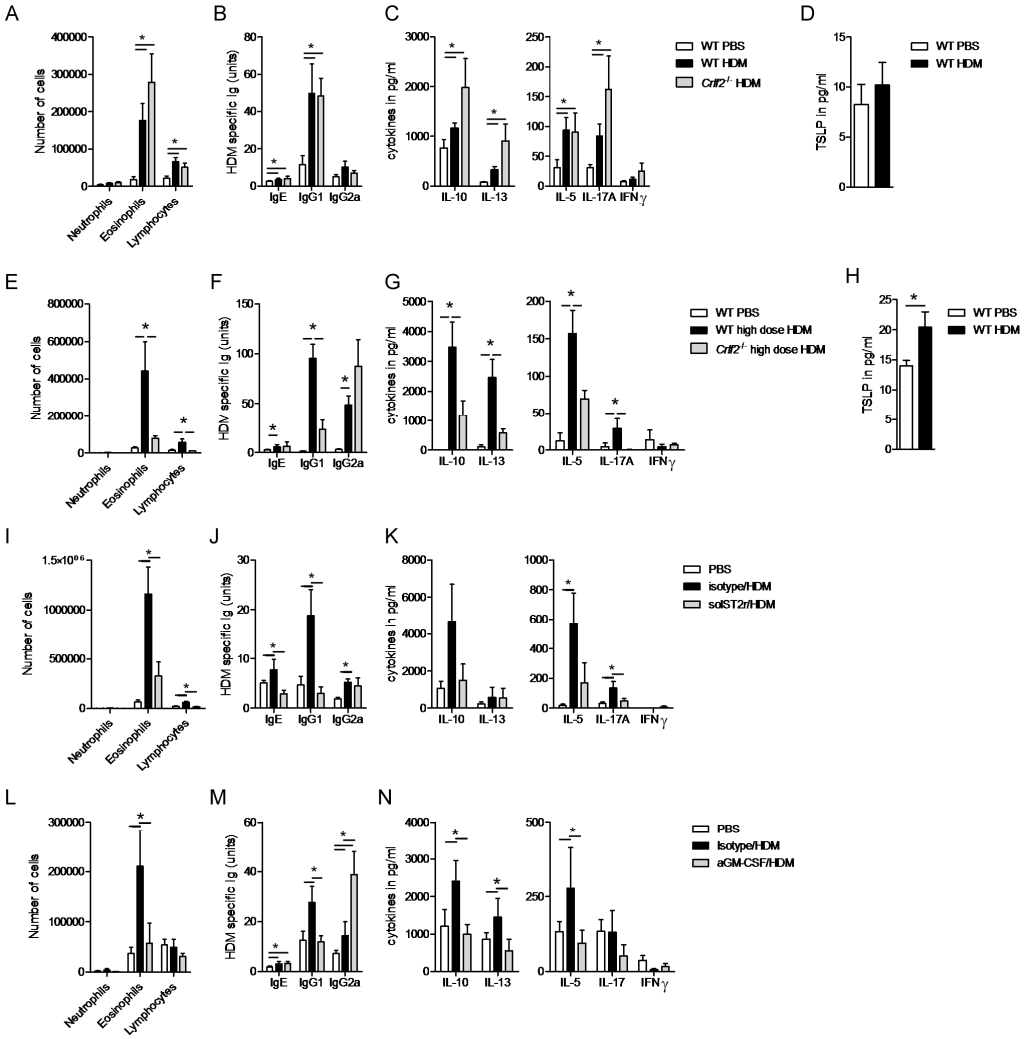

Figure 3. Development of Th_2 immunity to HDM depends on IL-33 and GM-CSF and not TSLP. (A–D) WT and *Crfl2*$^{-/-}$ were sensitized and challenged with HDM or PBS as described in Fig. 1. (**A**) Differential cell counts were determined 72 h later. (**B**) Levels of serum HDM–specific Igs. (**C**) Cytokine levels in MLN cells restimulated for 3 d with HDM. (**D**) TSLP levels in BAL fluids. (E–H) WT and *Crfl2*$^{-/-}$ were sensitized and challenged with 100 µg HDM or PBS. (**E**) Differential cell counts were determined 72 h later. (**F**) Levels of serum HDM–specific Igs. (**G**) Cytokine levels in MLN cells restimulated for 3 d with HDM. (**H**) TSLP levels measured in BAL fluids. (I–K) C57BL/6 mice were injected i.p. with blocking soluble ST2 receptor or isotype control at the time of HDM sensitization. (**I**) Differential cell counts were determined 72 h after the last HDM challenge. (**J**) Levels of serum HDM–specific Igs. (**K**) Cytokine levels in MLN cells restimulated for 3 d with HDM. (L–N) C57BL/6 mice were injected i.p. with blocking anti–GM-CSF or isotype control antibodies at the time of HDM sensitization. (**L**) Differential cell counts were determined 72 h after the last HDM challenge. (**M**) Levels of serum HDM–specific Igs. (**N**) Cytokine levels in MLN cells restimulated for 3 d with HDM. *, $P < 0.05$. Results show one representative experiment out of at least three. Five mice/group were used. Results are shown as mean ± SEM.

mice, *Crlf2*$^{-/-}$ mice developed less airway eosinophilia (**Fig. 3 E**) accompanied by a reduced Th_2 cytokine production by MLN cells (**Fig. 3 G**) and showed increased IgG_{2a} levels (**Fig. 3 F**), indicative of a switch to a Th_1 type of immunity. Moreover, exposure to a high dose of HDM was accompanied by an increase in BAL fluid levels of TSLP (**Fig. 3 H**). These findings suggest that the role of TSLP in asthma is related to severity of the disease, as it is in humans. Interestingly, when *Il1r1*$^{-/-}$ mice were administered the high dose of HDM, they still failed to develop asthma features (not depicted). To investigate the role of IL-33 in sensitization to low

dose HDM, we injected mice with recombinant soluble ST2 receptor (solST2r) to block IL-33 signaling. We found that the administration of solST2r at the time of sensitization led to a decrease in the number of eosinophils and lymphocytes in the BAL (**Fig. 3 I**) and in HDM-specific IgE and IgG_1 in the serum (**Fig. 3 J**). rST2 did not significantly affect the levels of cytokines produced by MLNs (**Fig. 3 K**). Because GM-CSF–deficient mice spontaneously develop alveolar proteinosis, we were not able to analyze the contribution of genetic deficiency of GM-CSF on HDM-driven asthma (Stanley et al., 1994). To address the functional importance of GM-CSF, we administered a neutralizing GM-CSF antibody at the time of low-dose sensitization to HDM. This lead to reduced eosinophilic influx in the lungs (**Fig. 3 L**), reduced HDM-specific IgG_1 (**Fig. 3 M**), and Th_2 cytokines in the MLNs (**Fig. 3 N**).

Administration of IL-1α is sufficient to promote sensitization to inhaled protein antigens

The aforementioned experiments demonstrated that IL-1α was necessary for inducing Th_2 immunity to HDM via activation of an epithelial cytokine cascade. As many parallel pathways might exist to induce Th_2 immunity to natural allergens in the lungs, we next wanted to study whether IL-1α was also sufficient to induce Th_2 immunity. To address this, we administered an otherwise tolerizing dose of harmless OVA antigen to the lungs of naive C57BL/6, in the presence or absence of rIL-1α. 10 d later, all mice were challenged with OVA aerosols. Addition of IL-1α resulted in a significant influx of eosinophils and lymphocytes in the BAL fluid compared with mice sensitized with only OVA or with PBS as a control (**Fig. 4 A**). Co-administration of OVA and IL-1α also induced increased levels of Th_2-dependent OVA-specific IgE and IgG_1 in the serum (**Fig. 4 B**), a Th_2 cytokine profile in MLNs (**Fig. 4 C**), GCM in lung ECs (**Fig. 4 D**), and bronchial hyperreactivity (**Fig. 4 E**). As in HDM-induced Th_2 immunity, IL-1 had a mild stimulatory effect on IL-17 production, whereas IFN-γ was not induced. These stimulatory effects of IL-1α on Th_2 immunity to OVA failed to develop in *Il1r1*$^{-/-}$ mice, suggesting that they were not caused by some off-target contaminating moiety in the cytokine (not depicted). Similar results were obtained when we administered OVA together with recombinant IL-1β, consistent with the idea that IL-1RI is stimulated equally well by IL-1α and IL-1β (**Fig. 3, A–D**). Interestingly LPS, an innate adjuvant known to promote Th_2 sensitization when administered at low dose, was able to increase bronchial hyperreactivity to the same extent as IL-1 (**Fig. 3 E**). We then addressed the relative contribution of TSLP and GM-CSF in mediating the Th_2-promoting effects of IL-1α. The induction of Th_2 immunity to OVA + IL-1α (as shown in **Fig. 4 F** for BAL fluid cellular composition, representative of all other parameters) was unaffected in *Crlf2*$^{-/-}$ mice lacking TSLPR. Moreover, the levels of TSLP measured in BAL fluids of the mice were very low and were not increased by IL-1α (not depicted). We also neutralized GM-CSF at the time of sensitization to OVA and IL-1α and subsequently challenged mice with OVA aerosol. Compared with mice receiving isotype antibody, GM-CSF neutralization reduced the influx of eosinophils and lymphocytes in the BAL fluid (**Fig. 4 G**), production of OVA-specific IgE and IgG_1 (**Fig. 4 H**), and Th_2 cytokines (**Fig. 4 I**).

IL-1 induces Th_2 sensitization via indirect effects on lung DCs

As eosinophilic airway inflammation, GCM, and allergen-specific Ig production are all controlled by CD4 T cell responses, we next addressed how mucosal IL-1 and IL-1R signaling

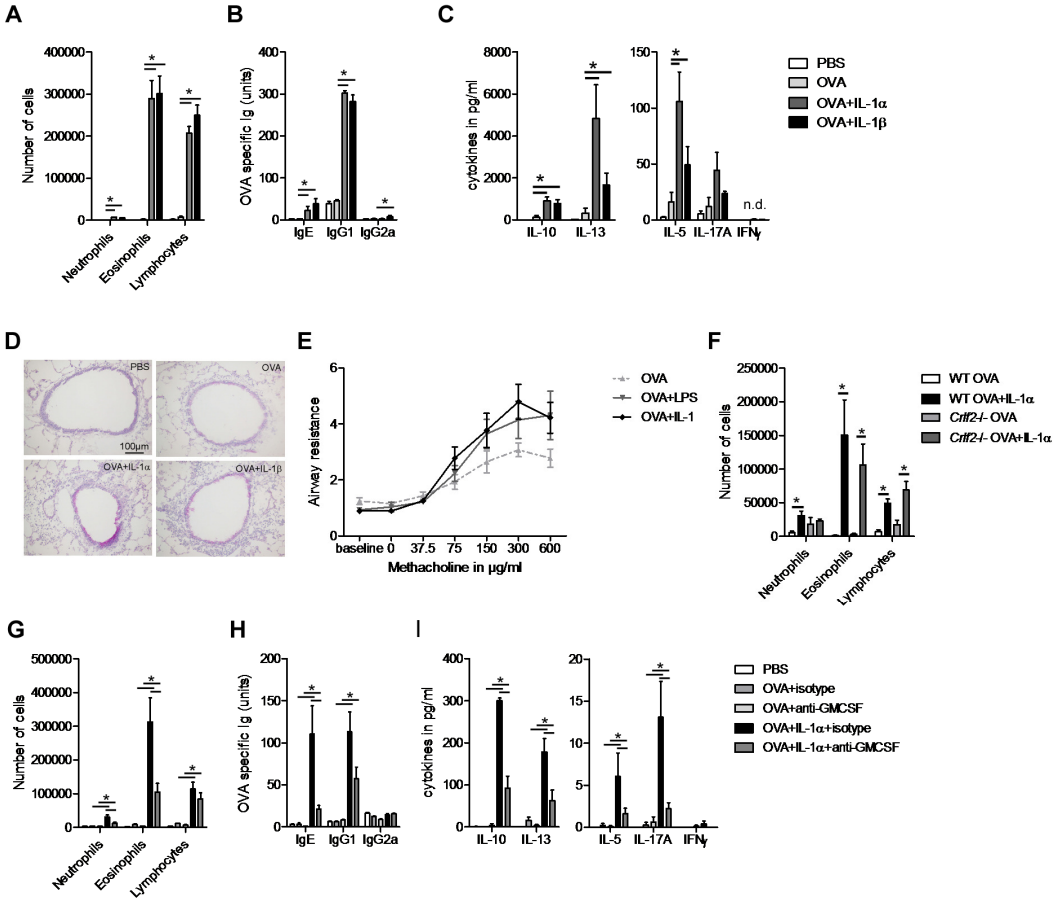

Figure 4. IL-1α is sufficient to promote sensitization to inhaled protein antigens. (A–E) C57BL/6 mice were sensitized on day 0 with OVA in the presence of IL-1α, IL-1β, or PBS. Mice were challenged with OVA aerosols on days 10–12. (**A**) Differential cell counts were determined 24 h after the last challenge. (**B**) Levels of serum OVA–specific Igs. (**C**) Cytokine levels in MLN cells restimulated for 4 d with OVA. (**D**) PAS staining of lung sections. (**E**) Bronchial hyperreactivity was analyzed in mice sensitized with OVA + IL-1 or OVA + LPS. (**F**) C57BL/6 and *Crlf2*$^{-/-}$ mice were sensitized with OVA or with OVA + IL-1α and were exposed to OVA aerosols. Differential cell counts were determined 24 h after the last challenge. (G-I) C57BL/6 mice sensitized with OVA in the presence or absence of IL-1α and injected with anti–GM-CSF or isotype control antibodies were challenged with OVA aerosols. (**G**) Differential cell counts were determined 24 h after the last challenge. (**H**) Levels of serum OVA-specific Igs. (**I**) Cytokine levels in MLN cells restimulated for 3 d with HDM. *, $P < 0.05$. Results show one representative experiment out of three. Five mice/group were used. Results are shown as mean ± SEM.

could affect CD4 T cell activation and polarization. For that reason, we injected the model antigen OVA and measured T cell responses in the MLNs after first transferring a cohort of CFSE-labeled OVA-specific TCR Tg OTII cells. As the IL-1RI is also expressed on CD4 T cells and IL-1 was shown to directly affect T cell activation when given systemically or subcutaneously, some mice received IL-1RI–deficient OTII T cells (obtained from *Il1r1*$^{-/-}$ × OTII mice). To eliminate the contribution of IL-1 on non-T cells and study whether mucosal IL-1 directly signals to T cells, WT OTII cells were also injected in *Il1r1*$^{-/-}$ recipient mice. 1 d later, we instilled i.t. OVA ± IL-1α or PBS as a control and studied T cell activation 3 d later. In WT recipient mice, OVA-specific T cells had divided more vigorously when IL-1α was injected together with OVA, regardless of whether the OTII cells were responsive to IL-1 or not (**Fig. 5 A**). However, this effect was not observed in *Il1r1*$^{-/-}$ recipient mice. **Fig. 5 B** shows increased IL-4, IL-5, and IL-17A expression in WT mice injected with WT or *Il1r1*$^{-/-}$ OTII cells. However, if the recipient was

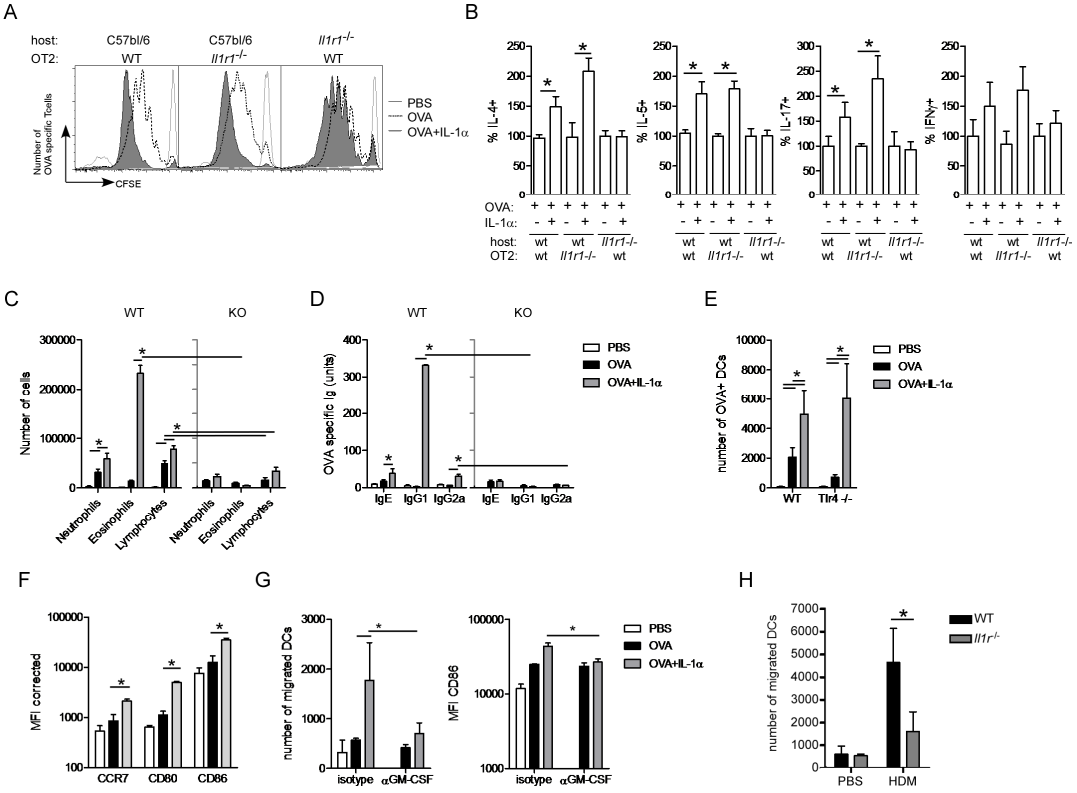

Figure 5. IL-1 induces Th_2 sensitization through indirect effects on lung DCs. (A and B) C57BL/6 and *Il1r1*$^{-/-}$ mice were injected with 10 × 10^6 CFSE-labeled OVA-specific WT OT2 cells or *Il1r1*$^{-/-}$ cells and were administered i.t. with PBS, OVA, or OVA + IL-1α. (**A**) Proliferation of CFSE-labeled T cells was determined by flow cytometry 4 d later. (**B**) Percentage of CFSE$^+$ T cells positive for IL-4, IL-5, IL-17A, and IFN-γ was determined by flow cytometry. (C and D) MHCII$^{fl/fl}$ × CD11c-Cre and WT mice were sensitized with OVA or OVA + IL-1α and were challenged with OVA aerosols. (**C**) Differential cell counts were determined 24 h after the last challenge. (**D**) Levels of serum OVA–specific Igs. (**E**) WT and Tlr4$^{-/-}$ mice were administered fluorescent OVA i.t. The number of migrating OVA$^+$ MHCII^{hi}CD11c$^+$ DCs was determined in the MLNs 24 h later. (**F**) WT mice were administered i.t. with OVA, OVA + IL-1α, or PBS as a control. The levels of expression of CCR7, CD80, and CD86 were determined using flow cytometry. (**G**) WT mice were administered OVA i.t., OVA + IL-1α, or PBS. Mice were also injected with blocking antibodies to GM-CSF at the time of sensitization. The number of migrating MHCII^{hi}CD11c$^+$ DCs and their levels of CD86 expression were determined in the MLNs 24 h later. (**H**) WT and *Il1r1*$^{-/-}$ mice were administered HDM or PBS. The number of MHCII^{hi}CD11c$^+$ DCs was determined in the MLNs 24 h later. *, P < 0.05. Results show one representative experiment out of at least two experiments. Four to five mice/group were used. Results are shown as mean ± SEM.

deficient of IL-1RI, these cytokines were not increased above the level seen in mice receiving only OVA. These data show that mucosal IL-1α promotes Th_2 immunity not by directly affecting antigen-specific CD4 T cells. As inhaled OVA is presented to CD4 T cells mainly by lung DCs and given the fact that CCL20 is a prototypical chemokine attracting immature DCs to the lungs and GM-CSF is an important development and maturation cytokine for DCs, we next studied whether IL-1 and the downstream cytokine cascade promoted development of Th_2 immunity by promoting the antigen-presenting function of lung DCs. To address this, we administered OVA + IL-1 to mice in which CD11c$^+$ DCs do not express MHCII (MHCII$^{fl/fl}$ × *Cd11c*-Cre$^+$). MHCII$^{fl/fl}$ × *Cd11c*-Cre$^-$ littermates served as controls. In control mice, OVA + IL-1 induced eosinophilia and lymphocytosis (**Fig. 5 C**), as well as serum Th_2–dependent OVA-specific IgG_1 (**Fig. 5 D**), Th_2 cytokines in MLN cultures (not depicted), and GCM (not depicted). These features of asthma were strongly reduced in mice lacking MHCII on CD11c^{hi} cells. We finally

wanted to address whether and how IL-1α affected DC function and activation *in vivo*. To trace DC migration, we injected mice with fluorescently labeled OVA with or without IL-1α. 1 d later, the MLNs were analyzed for the number of OVA-carrying DCs and their degree of maturation. Compared with OVA alone, the addition of IL-1α to OVA resulted in enhanced migration of DCs to the MLNs and increased expression of maturation markers CD80 and CD86 on the migrated DCs (**Fig. 5, E and F**). The effect was also observed in *Tlr4*$^{-/-}$ mice, demonstrating that the effect of IL-1 was not caused by endotoxin contamination in the recombinant IL-1α. Enhanced DC migration and increased expression of maturation markers were inhibited if GM-CSF was neutralized at the time of OVA + IL-1α injection (**Fig. 5 G**). To study whether DC functions were also affected by endogenous IL-1R, we studied the migration of lung DCs to the MLNs in WT and *Il1r1*$^{-/-}$ mice exposed to HDM, a trigger for IL-1α release. As shown in **Fig. 5 H**, HDM administration led to enhanced DC migration to the MLNs, an effect strongly reduced in *Il1r1*$^{-/-}$ mice.

Discussion

In this study, we have uncovered a crucial role for IL-1R and IL-1α in causing Th_2 sensitization to inhaled HDM. The precise role of IL-1α and IL-1β in development of allergy has been unclear and studied mainly using the model antigen OVA. In some experiments in which OVA alum was injected i.p., there was no role for either cytokine as *Il1r1*$^{-/-}$ mice developed all features of asthma (Schmitz et al., 2003). However, the administration of recombinant IL-1α at the time of OVA/alum sensitization was shown to reduce asthma features (Caucig et al., 2010). In that study, IL-1α administration at later time points exacerbated the disease. In a milder model in which sensitization was induced in the absence of alum in *Il1r1*$^{-/-}$ mice, asthma was reduced (Schmitz et al., 2003). The most logical conclusion from these conflicting data is that the strength of model, the route of administration, and the cell population targeted might determine the requirement for IL-1 in disease development. The i.p. injection of IL-1α primarily targets peritoneal macrophages and induces Th_1 responses that can suppress Th_2 immunity (Caucig et al., 2010). In our study, the i.t. administration of the cytokine preferentially triggers lung ECs, thus favoring Th_2 immunity. Using radiation bone marrow chimeric mice and exploiting the natural route of pulmonary exposure to HDM, we provide evidence that IL-1R triggering on radioresistant lung ECs promotes the innate immune response to natural allergens, a feature which was also observed in cigarette smoke–exposed mice (Botelho et al., 2011). Development of Th_2 immunity to inhaled HDM or model antigens requires triggering of the TLR4 receptor on radioresistant cells (Hammad et al., 2009; Tan et al., 2010; Kool et al., 2011). This induces the release of epithelial cytokines (TSLP, GM-CSF, IL-33, and IL-25) and chemokines (KC and CCL20) that promote Th_2 immunity by activating basophils, eosinophils, and DCs (Fort et al., 2001; Hurst et al., 2002; Cates et al., 2004; Saenz et al., 2008; Besnard et al., 2011). We have set up ALI cultures of primary ECs from the conducting airways of mice and confirmed that GM-CSF, IL-1α, and KC were produced by HDM-stimulated ECs in a process requiring TLR4. We did not find consistent induction of TSLP, IL-33, and IL-25 in the ALI cultures, which does not exclude a role for them *in vivo*. Strikingly, the production of GM-CSF was impaired in ALI cultures set up from *Il1r1*$^{-/-}$ mice, uncovering the presence of an endogenous autocrine loop of IL-1α acting on IL-1RI on ECs to promote

cytokine production. We also found evidence for this innate pro-Th_2 amplification loop *in vivo*, as exposure of *Il1r1*$^{-/-}$ mice to HDM led to significantly reduced production of GM-CSF, IL-33, and KC in lung homogenates, suggesting an important role for endogenous IL-1 in promoting Th_2 immunity to allergens. Conversely, when we administered IL-1α to naive mouse lungs *in vivo*, we could induce production of GM-CSF and CCL20 (unpublished data). We have recently reported that endogenous danger signals like UA or ATP played an important role in the process of Th_2 sensitization (Kool et al., 2011). In this study, we show that HDM-induced UA production was required for IL-1α production. However the neutralization of ATP with apyrase did not affect IL-1α secretion *in vivo*, indicating that ATP might be downstream of IL-1α production or might be involved in a different pathway. There are some subtle differences between the induction of cytokines and chemokines in the ALI cultures *in vitro* and the response seen *in vivo*. IL-33 was not induced by HDM *in vitro* but was consistently induced *in vivo* in a process requiring IL-1RI. This could be a potentially important observation, as IL-33 has been shown to promote Th_2 immunity to allergens by stimulating the function of DCs and innate lymphoid cells (Besnard et al., 2011; Eiwegger and Akdis, 2011). We have previously reported that Th_2 immunity induced by inflammatory DCs is blocked when IL-33 signaling is blocked by administration of the soluble T1/ST2 receptor (Lambrecht et al., 2000). In support, we also observed a decrease in the number of BAL eosinophils and HDM-specific Igs when IL-33 signaling was blocked at the time of sensitization in this HDM model. A central role has been attributed to TSLP in the process of allergic sensitization (Zhou et al., 2005; Liu et al., 2007; Headley et al., 2009). Several groups have reported that IL-1 can induce TSLP (Allakhverdi et al., 2007; Lee and Ziegler, 2007), and a recent study demonstrated that lung DCs produce TSLP upon exposure to HDM (Kashyap et al., 2011). We found that the development of Th_2 immunity driven by IL-1 or by a low dose of HDM did not require TSLP or its receptor. When a high dose of HDM (100 μg) was used to sensitize and challenge mice, airway eosinophilia was reduced in mice lacking TSLPR. Exactly why there is this allergen dose–dependent effect of TSLP is unclear at present. In the high-dose model, IgG_{2a} levels were increased, indicative of a mixed Th_1 and Th_2 response. As Th_1 responses can counteract Th_2 immunity, we speculate that the absence of TSLP or of its receptor further shifts the immune response to Th_1 response. In the low-dose model, the threshold for Th_1 induction might be too high for TSLP blockade to have this enhancing effect. GM-CSF is a hematopoietic cytokine associated with Th_2 immunity in the lung (Stämpfli et al., 1998; Ohta et al., 1999; Cates et al., 2004; Bleck et al., 2006). This cytokine was reliably induced *in vitro* and *in vivo* by both HDM and IL-1α. Not surprisingly, neutralization of GM-CSF at the time of sensitization to HDM or OVA + IL-1α *in vivo* strongly reduced the salient feature of asthma. Human bronchial ECs have been shown to make IL-1α and IL-1β in an asthmatic setting and also express the IL-1RI (Mattoli et al., 1990; Marini et al., 1991). Our findings on cultures of human ECs demonstrated that IL-1α was increased after HDM exposure and that IL-1α could induce GM-CSF. It is therefore likely that the pathway that we discovered is also operative in humans exposed to allergens and could also help explain the adjuvant effects of air pollutants (Bleck et al., 2006). In the current study, we identify IL-1α and not IL-1β as the predominant IL-1 cytokine driving the innate cytokine cascade, but we have not studied how IL-1α is produced. IL-1α can be released as a cytokine by inflammatory cells or as an alarmin by dying cells (Chen et al., 2007; Dinarello, 2009). We have found it to be produced by bronchial ECs in ALI cultures and by

alveolar macrophages early in the response to HDM (unpublished data). However, we have never detected dying cells after HDM administration (unpublished data), but more studies are required to rule out the possibility that dying cells contribute to IL-1α release. Experiments with *casp1*$^{-/-}$ mice did not support a role for IL-1β in Th_2 immunity, in line with a previous publication on the lack of involvement of the NLRP3 inflammasome in HDM-driven asthma (Kool et al., 2011). However, various papers show that IL-1β could be cleaved to its active form outside the cell by enzymes (proteinase3 and elastase) secreted by neutrophils (Joosten et al., 2009). We found no significant reduction in HDM-induced Th_2 immunity when neutrophils were depleted using antibodies at the time of sensitization (unpublished data). DCs are necessary and sufficient to cause Th_2 immunity to HDM (Hammad et al., 2010). We found that the type of Th_2 immunity induced by IL-1α relied completely on antigen presentation by DCs and that IL-1α induced the migration and activation of DCs in a GM-CSF–dependent way. Clearly, the process of Th_2 development induced by mucosal administration of IL-1 or the enhanced proliferation of antigen-specific T cells seen did not require direct signaling of IL-1RI on T cells, as Th_2 immunity was still induced when OVA and IL-1 were administered to the lungs of mice harboring OVA-specific TCR Tg T cells lacking IL-1RI. Strikingly, the same observation was made for induction of Th_{17} responses in the lung-draining nodes, which did not require IL-1RI on T cells. This is in contrast to studies in which IL-1 was given systemically or subcutaneously and promoted proliferation and Th_2/Th_{17} immunity by directly acting on T cells (Sutton et al., 2006; Ben-Sasson et al., 2009). One explanation could be that administration to the lung does not allow sufficient IL-1α to reach the T cells. In our opinion, the promotion of enhanced T cell proliferation and Th_2 immunity is best explained by effects of IL-1α and GM-CSF on DCs, which subsequently promote T cell expansion and differentiation. In parallel, other cytokines like IL-33 that are simultaneously induced could affect T cells directly. In conclusion, our experiments in mice and human bronchial ECs have unraveled a new mechanism that helps explain sensitization to HDM allergen. HDM triggers ECs to produce IL-1α in a TLR4-dependent manner. The IL-1α subsequently acts in an autocrine manner on the lung ECs, leading to secretion of pro-inflammatory chemokines, GM-CSF, and IL-33. Together, these recruit and activate inflammatory DCs that induce adaptive Th_2 immunity to the allergen. It will be interesting to study whether this cascade of innate cytokines programming adaptive immunity is induced by other natural allergens, environmental pollutants, and respiratory viruses that enhance the inflammatory response.

Materials and methods

Mice. TSLP receptor$^{-/-}$ (*Crlf2*$^{-/-}$) mice on C57BL/6 background (backcrossed for at least 10 generations to C57BL/6) were provided M. Comeau (Amgen, Thousand Oaks, CA); *Il1r1*$^{-/-}$ mice (backcrossed for 8 generations to C57BL/6) were provided by B. Ryffel (University of Orléans, Orléans, France); *Casp1*$^{-/-}$ mice were provided by T. Vandenberghe (Flanders Institute for Biotechnology, Zwijnaarde, Belgium); and *Mhc2*$^{fl/fl}$ *CD11c*-Cre^{+} and Mhc2$^{fl/fl}$ CD11cCre^{-} mice were provided by A. Liston (University of Leuven, Leuven, Belgium; backcrossed for at least 10 generations to C57BL/6). Female C57BL/6 WT mice were obtained from Harlan. *Tlr4*$^{-/-}$ (backcrossed for eight generations to C57BL/6) mice and MHCII-restricted OVA-TCR Tg OTII mice were obtained from the Jackson Laboratory. Mice were housed under specific pathogen–free conditions in individually ventilated cages in a controlled day–night cycle and given food and water ad libitum. All experiments were approved by the animal ethics committee of Ghent University.

Reagents. HDM extracts were obtained from Greer Laboratories. Recombinant soluble ST2 was provided by H. Braun (Flanders Institute for Biotechnology). Rasburicase (uricase) was purchased from Fasturtec, and human IL-1ra

(anakinra) was obtained from Amgen. For sensitization of mice, endotoxin low OVA was obtained from Hyglos and Worthington Biochemicals, whereas for use during antigen challenge, Grade III OVA and apyrase were obtained from Sigma-Aldrich. We obtained purified anti–IL-1α, recombinant mouse and human IL-1α, IL-1β, and IL-33, and ELISA Duoset for mIL-13, mKC, mCCL20, mIL-25, mIL-33, hIL-1α, hIL-1β, hIL-33, hTSLP, and hGM-CSF from R&D Systems. ELISA to detect hIL-25 was obtained from Wuhan EIAab Science. FITC-labeled antibody to MHCII, PE-Cy^5–labeled antibodies to CD3 and CD19, PE-Cy^7–labeled antibody against CD49b, and APC-labeled antibody to CD11c, MHCII, and F4/80, and ELISA sets for mMCP1, mTSLP, and mIL-17A were acquired from eBioscience. FITC-labeled antibody to Ly6C, PE-labeled antibody against Siglec-F and Ly6G, Horizon V450–labeled antibody against CD11b, as well as mIL-1α, mIL-1β, mIL-4, mIL-5, mIL-10, mIFN-γ, and mGM-CSF ELISA sets and antibody pairs to mouse IgE, IgG_1, and IgG_{2a} to measure Igs in serum were obtained from BD. Aqua, CFSE, PE–Texas red–labeled antibody against CD11c and OVA Alexa Fluor 488 were purchased from Molecular Probes/Invitrogen. PerCp-$Cy^{5.5}$–labeled antibody against MHCII and LEAF-purified anti–IL-1α, anti–IL-1β, anti–GM-CSF, and isotype control antibodies for neutralization experiments were obtained from BioLegend.

Generation of bone marrow chimeras. 8–10-wk-old *Il1r1*$^{-/-}$ and WT mice were sublethally irradiated (8 Gy) and received 2 × 10^6 bone marrow cells i.v. from either *Il1r1*$^{-/-}$ or WT C57BL/6 donors 4 h after irradiation. Mice were used in experiments at least 10 wk after bone marrow reconstitution.

Model of HDM-induced asthma. Mice were anesthetized with isoflurane and received 1 µg HDM intranasally 7 d later, mice were challenged with 10 µg HDM on five consecutive days under anesthesia. 3 d after the last challenge, mice were sacrificed and organs were dissected for analysis. BAL was performed using 3× of 1 ml EDTA-containing PBS and analyzed, and lungs were inflated with PBS/OCT (1:1) solution and snap frozen in liquid nitrogen and kept at -80°C until further processing. Blood was taken to collect serum and analyzed for HDM-specific Igs. MLNs were dissected, and single cell suspensions were prepared by pressing through a 100-µm cell sieve and restimulated in vitro with 15 µg/ml HDM for 3 d. Supernatant was collected from these cultures, and cytokine profiles were assayed by ELISA. Lung function was performed using Flexivent invasive measurement of dynamic resistance as described previously (Hammad et al., 2007). In neutralization experiments, IL-1α and IL-1β were blocked at the time of sensitization to HDM by use of 70 µg blocking antibody injected i.t. In other experiments, 150 µg anti–GM-CSF antibody or 200 µg recombinant soluble ST2 was injected i.p. at the time of sensitization.

Early innate immune response to HDM. Mice were i.t. instilled with PBS or 100 µg HDM. Apyrase- and uricase-treated mice were injected i.t. with 4 U/ml apyrase or 100 µg uricase 30 min before exposure to PBS or HDM. Mice were injected i.p. with 200 µg recombinant soluble ST2 (rST2). Mice were sacrificed 2 or 24 h after the injection. BAL was collected, and the left lung was snap frozen in liquid nitrogen and stored at -80°C for preparation of lung homogenates. It was then homogenized with a tissue homogenizer in 500 µl of cold lysis buffer (20 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 10% glycerol [vol/vol], 1 mM PMSF, 1 mM sodium orthovanadate [Na3VO4], 1 µM NaF, 40 mg/ml aprotinin, and 20 mg/ml leupeptin) using a tissue homogenizer (IKA) with the addition of 1% Igepal after homogenization. Samples where then kept on ice for 30 min with agitation each 10 min, followed by a centrifugation to pellet debris. Cleared lysate was quantified for protein concentration with NanoOrange reagent (Invitrogen) according to the manufacturer's protocol. Cell suspensions were made of the right lung and used for FACS analysis. Innate cytokines were measured by ELISA on lung homogenates, and concentrations were corrected for the protein content.

OVA experiments. DC migration was investigated by injecting 100 µg OVA Alexa Fluor 488 i.t. and dissecting MLNs after 24 h. Cells were stained for flow cytometry, and DCs positive for Alexa Fluor 488 and expressing high levels of MHCII and CD11c were considered to be migratory DCs. For i.t. administration of PBS, 100 µg OVA-w ± 80 ng IL-1α, 80 ng IL-1β, 80 ng LPS, and 8 or 80 ng IL-33 mice were anesthetized with isoflurane at day 0 and 1. At day 10, mice were challenged with OVA aerosols (1% OVA grade III solution) for 30 min on three consecutive days. Mice were sacrificed 24 h after the last aerosol. BAL was performed and cells were analyzed as described previously (van Rijt et al., 2004). MLNs were dissected and restimulated for 4 d with 10 µg/ml OVA-w. Supernatant was harvested at day 4 and analyzed for cytokines by ELISA. For T cell division experiments, lymphocytes from LNs and spleen from OTII TCR Tg mice were isolated and stained with CFSE and injected i.v. in recipient mice. 1 d later, mice received an i.t. injection of PBS, 100 µg OVA-Endograde ± 80 ng IL-1α or 80 ng IL-1β. After 4 d, the MLNs were analyzed for T cell divisions and cultured for 3 d for cytokine response, without restimulation. Flow cytometry was performed on LSRII (BD).

ALI cultures of mouse and human tracheal ECs. The isolation and culture of tracheal ECs were performed with small adaptations as previously described (Mayer et al., 2008). 8-wk-old mice were sacrificed with CO_2, and trachea were dissected free and digested with pronase E and DNase I overnight at 4°C. Cell suspensions were allowed to adhere for 2 h in a Petri dish at 37°C. Non-adherent cells were grown for 4–7 d until confluence was reached (>5 kOhm, measured by transepithelial resistance) in a transwell system on collagen (Sigma-Aldrich)-coated membranes

(Corning). These cells were cultured for 3 wk as ALI cultures and subsequently exposed for 24 h to 100 µg HDM, 10 ng IL-1α, or 10 ng IL-1β or PBS as a control. Cytokines secreted in medium were measured by ELISA. Human NBECs were purchased from Lonza. Cells were cultured in T80 culture flask (Thermo Fisher Scientific) to expand the cell numbers in BEGM growth medium as proposed by the manufacturer, until $^{+}/_{-}$ 85–90% confluence of cells. Cells were harvested and plated on collagen (Sigma-Aldrich)-pre-coated transwells (Corning) until cells reached confluence. Medium on the apical side was removed, and medium at the basolateral side was replaced by B-ALI growth medium (Lonza). Cells were cultured for 4 wk in B-ALI growth medium and pulsed overnight with PBS, 100 µg HDM ± IL-1ra, or 10 ng recombinant hIL-1α. Cytokine levels were measured in supernatant by ELISA.

Statistical analyses. For all experiments, we calculated the difference between groups with the Mann-Whitney U test for unpaired data (SPSS version 15.0). Differences were considered significant when the p-value was <0.05.

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Recent findings

In an OVA-tolerance model of allergic asthma HDM was able to break tolerance if administered prior to sensitization (Soroosh et al., 2013). In this study, alveolar macrophages were isolated and were shown to secrete high amounts of IL-1α if tolerance was blocked. In our model of HDM-induced allergic asthma, blocking IL-1α during the secondary immune response did not modify the features of asthma (figure 1I). It would be interesting to investigate if alveolar macrophages in this model secrete IL-1α during the challenge phase. Since alveolar macrophages were demonstrated to induce airway tolerance. This raises the hypothesis that IL-1α is only relevant during the sensitization phase, since blocking IL-1α during the secondary immune responses did not reduce allergic airway inflammation.

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General discussion and prospects for the future

General discussion and prospects for the future

In this thesis, we have shown that the activation of the pulmonary immune response is tightly controlled by interaction between airway epithelial cells and dendritic cells, occurring via release of paracrine cytokines and danger signals. Broadly speaking, DCs can be divided into different subsets which are found in different tissue compartments. Most is known about the DC subsets that line the conducting airways, and are involved in the recognition of inhaled microbes, pollutants and allergens [151]. Recently, our group has found that the main subsets involved allergic asthma are mo-DC and $CD11b^+$ cDC [19, 152, 153]. Mo-DCs are able to present antigen to $CD4^+$ T cells, however to a lesser extent than $CD11b^+$ cDCs. Both subsets can initiate sensitization to HDM after adoptive transfer of mice exposed to HDM. In models where low doses of HDM are used to induce sensitization, $CD11b^+$ DCs migrate to the MLN and present allergens to naive T cells. Recruited mo-DCs are mainly responsible for producing chemokines and cytokines that drive Th_2 responses during ongoing inflammation, and for local restimulation of Th_2 effector cells through expression of costimulatory molecules [13]. Not surprisingly, depletion of DCs during secondary challenge to allergens abolishes the cardinal features of asthma [8]. Interestingly, using higher doses of HDM for sensitization, led to recruitment of mo-DCs to the lung which subsequently migrated to the MLNs and had the potential to also induce sensitization. In models of viral infection, work to which I contributed, found that $CD103^+$ cDCs have distinct functions and are very important for cross-presentation to CD8 T cells [10], while the $CD11b^+$ DCs are mainly important for inducing tertiary lymphoid tissues that maintain local Ig synthesis [154]. The origin and general function of airway lining dendritic cells is summarized in **chapter 1**.

In **chapter 3**, we have studied one of the most unknown DC subsets of the lungs, that lines the lung blood vessels in the lung interstitium. Although there is very little primary research on this topic, it has always been assumed that lung interstitial DCs are poorly migratory and only contribute to secondary immune responses. We here show that the lung interstitium has a warning system made up of cDCs lining the blood vessels and alerting of foreign embolic material, leading to recruitment of monocytes. We found that vessel lining interstitial DCs recruit inflammatory monocytes by releasing MCP-1 as soon as foreign embolic antigen was trapped in the lung-vascular bed. Interstitial DCs are not likely to migrate to the MLN [12]. The monocytes develop into DCs that migrate to the MLN and prime for the induction of a T cell response directed to antigens found in the embolic material. These T cells return to the site where the embolus was trapped and cause a short-lived inflammation. We can only speculate why this form of inflammation develops in response to a sterile stimulus (Sepharose beads coated with OVA antigen), even when addition of LPS increased inflammation. Upstream of the activation of interstitial DCs, activation of endothelial cells may occur since large foreign material leads to disruption of the blood flow in vessels, potentially causing endothelial damage. Vascular endothelial cells are exposed to fluid shear stress of a magnitude and pattern that varies throughout the vascular tree. These stress conditions alter the inflammatory responses of endothelial cells, particularly their ability to recruit leukocytes [155]. Therefore it is very likely, that these stressed endothelial cells secrete cytokines

providing an inflammatory micro-milieu, in which surrounding tissue cDCs get activated. In their turn activated cDCs secrete MCP-1 recruiting $CCR2^+$ monocytes or mo-DCs. We did not use the markers FcγRI (CD64) and FcεRI (MAR-1) to distinguish mo-DCs and $CD11b^+$ cDCs, since those markers were not described at the time we performed these studies. Our observations were based on CX3CR1, CD11b and Ly6C expression, however the $Ly6C^{hi}$ mo-DCs could be the same population as $CD64^+$ monocytes. $CD64^+$ monocytes gain MHCII expression and lose their $Ly6C^+$ expression while becoming a functional DC [28, 156]. CD11b and Ly6C were also used to sort monocytes from the bone marrow and transfer them into DC-depleted mice. Adoptive transfer of these monocytes restored the embolic antigen presentation in the MLN. We observed that only proper stimulation of DCs occurred if the antigen (coated on the beads) was co-administered with endotoxin and anti-CD40L. Mo-DCs were then capable of initiating proper CTL responses. These results show embolic antigens are presented in the lung draining lymph nodes. The main DC subset involved in antigen presentation, $CD11b^+$ cDCs or mo-DCs, still remains to be investigated using markers CD64 and MAR1. We also need to further research why the inflammation around the Sepharose beads is so short lived, despite the long persistence of antigen on the beads and despite provision of LPS and CD40L signals. Unraveling the reasons behind this rapid downregulation of the pulmonary immune response in the lung interstitium might reveal critical insights in the future as to the pathogenesis of interstitial lung diseases like sarcoidosis, also accompanied by temporary lung infiltrates and swollen mediastinal lymph nodes.

In the past, our group has studied extensively how we can modulate the function of DCs using pharmacological compounds, and many of these interfere with natural metabolites that also influence the basic physiology of DCs. As an example, interfering with the function of sphingosine-1-P by use of FTY720 was able to suppress the salient features of asthma [157]. In **chapter 4**, we show that the activation of DCs in the lung can be suppressed by the bile acid compound UDCA. In humans, the main primary bile acids are cholic acid and chenodeoxycholic acid (CDCA) and the principle secondary bile acids are deoxycholic acid and lithocholic acid. The natural occurring ursodeoxycholic acid (UDCA), is an endogenous mediator mainly present in low amounts in serum as its taurine conjugate tauroursodeoxycholic acid (TUDCA) [158]. UDCA is used in the clinic to treat patients with chronic cholestatic diseases [159]. We found that UDCA has the potential to suppress airway inflammation. UDCA has immunosuppressive properties by acting on the DCs directly via the farnesoid X receptor (FXR), but the downstream signaling pathways effected by UDCA administration still need to be investigated. The FX receptor is mainly expressed on hematopoietic cells [160]. CDCA can bind the same receptor but did not lead to a decrease in inflammation. Deoxycholic acid and CDCA were found to be cytotoxic to T cells and DCs. Binding of bile acids to FXR can lead to induction of small heterodimer partner (SHP) within the cytoplasm, although only in combination with retinoids [161]. SHP has been recognized as a negative regulator in intracellular signaling which is shown in allergic airway inflammation and is expressed predominantly in hematopoietic cells [162, 163]. It has been shown to be negatively regulating CD64 and MAR-1 which are both expressed on inflammatory DCs [28, 164, 165]. The inhibition of these receptors occurs by preventing tyrosine phosphorylation of the receptor and Syk, as well as the increase of intracellular calcium release [166]. TUDCA is also a known inhibitor of endoplasmatic reticulum

(ER) stress, through its capacity to act as a protein folding chaperone molecule. Perhaps inhibiting ER stress signaling leads to reduced inflammation in the model of allergic asthma. Polymorphisms in a genetic locus containing ORMDL3 is the only genetic risk factor recently associated to asthma in a genome wide association study (GWAS). ORMDL3 is expressed in hematopoietic and stromal cells, and alters ER-mediated Ca^{2+} homeostasis and facilitates the unfolded-protein response (UPR) [167-169]. The ER protein anterior gradient homolog 2 (AGR2) is localized to the ER of MUC5AC- and MUC5B-producing airway epithelial cells and forms complexes with immature MUC5AC. Expression of AGR2 and MUC5AC in airway epithelium from asthmatics is simultaneously increased. Allergen challenged Agr2 deficient mice have impaired mucin production accompanied by increased proportion of mucins contained within the ER leading to ER stress in airway epithelium [170]. Reducing ER stress could lead to less inflammation, however direct experiments with TUDCA have never been performed. One other possibility could be, as shown in a liver transplantation study, that protective effects of TUDCA are mediated through a mechanism involving proliferator-activated receptor-γ (PPARγ) signaling [171]. The FXR is identified as a modulator of lipid and glucose metabolism and PPARs are activated by arrays of polyunsaturated fatty acid derivatives, oxidized fatty acids and phospholipids. Our group showed in allergic asthma that PPARγ activation on DCs prevents induction of Th_2-dependent eosinophilic airway inflammation and contributes to immune homeostasis in the lung [172]. In allergic asthma PPARγ activation by TUDCA on DCs is possible, but needs further investigation. Inhibition of CD64 and MAR-1 signaling pathways are very likely to happen since we also found a role for Syk signaling on dendritic cells dependent on UA activation (**chapter 5**). However we have not investigated if UA levels are reduced by UDCA treatment. Chapter 4 shows that DC activation in the lung is a very tight control point that is implicated in driving Th_2 responses to allergens.

Uric acid crystals were shown to directly engage cholesterol-rich cellular membranes of DCs in a receptor-independent manner [173]. In **chapter 5**, we have further elaborated on the idea that endogenous danger signals, released by bronchial epithelial cells are crucial in controlling the activation state of DCs in response to allergens. Injection of inert allergens such as OVA combined with UA lead to *in vivo* development of Th_2 cell immunity. The early activation and recruitment of inflammatory DCs upon UA exposure was mediated by Syk activation and PI3Kδ signaling in DCs (chapter 5). DAMPs such as monosodium urate crystals and ATP are described to activate the NALP3 inflammasome. Recently also reactive oxygen species (ROS) were proposed to cause potassium efflux and thereby inflammasome activation (and vice versa) [174]. Although current studies implicated ROS exposure leading to NF-κB upregulated expression of NLRP3 and pro-IL-1β transcripts instead of inflammasome activation (reviewed in [215]). Surprisingly, mice deficient of any of the IL-1β mediated pathways (NALP3, ASC or IL-1RI) still initiated Th_2 responses to OVA mixed with UA crystals. We also found increased UA upon inhalation exposure to HDM in lung lavage fluid of mice, which was diminished in TLR4 deficient mice. Furthermore real life allergens, like HDM, lead to increased levels of UA in bronchoalveolar lavage fluid of allergic asthmatics. To investigate the role of UA in sensitization to HDM we used uricase, an enzyme degrading UA. Uricase-treatment at sensitization to HDM led to reduced eosinophilic influx and Th_2 responses. We have shown that allergic asthma to HDM is dependent on TLR4 signaling on lung stromal cells [55]. As

depicted in **chapter 1**, lung epithelial cells release several cytokines upon HDM exposure. The most pronounced AEC cytokines released were also abrogated by the addition of uricase, like GM-CSF, TSLP and IL-25. Confocal images of lungs taken after HDM exposure revealed that AECs are the source of UA. Complex allergens such as HDM can activate multiple pathways simultaneously. Since uric acid and these cytokines can be found at the same time after allergen exposure, it is very likely that these multiple pathways exist in parallel [88]. Close interaction between epithelial cells and tissue DCs leads to activation of DCs by UA and exposure to Th_2 inducing cytokines like GM-CSF and IL-25. UA release is reduced in mice deficient of TLR4, but not in IL-1RI deficient mice. Suggesting that UA release is downstream of TLR4, but upstream of IL-1RI. However, IL-1RI deficient mice were responding to OVA+UA crystal sensitization like control mice, suggesting that UA crystals can also boost Th_2 immunity via other pathways. This correlates well with experimental asthma models where OVA was mixed with alum, and in which also the IL-1RI deficient mice developed asthma [149].

In addition, injection of pro-inflammatory IL-1β lead to a small increase in UA in the lavage of mice, in contrast to IL-1α exposure, however the levels were as high as HDM. It raises again the possibility that multiple pathways exist in the sensitization to HDM. However we could not find a role for IL-1β in allergic asthma, nor in caspase1 of Nlrp3 deficient mice which are elements upstream of IL-1β to cleave IL-1β into its bioactive form (**chapter 1 and 5**).

We found that TLR4 and interleukin 1-receptor 1 (IL-1RI) signaling (**chapter 6**) on stromal cells are both important in the sensitization to HDM [55]. Both pathways could therefore be a target in therapeutics against asthma. However TLR4 is used to recognize bacterial infections in the lung, since its well-known ligand is lipopolysaccharide (LPS). Blocking the signaling of this pathway may induce problems in clearing lung infections. The IL-1/IL-1RI pathway has been shown to be involved in other lung diseases like lung fibrosis and chronic obstructive pulmonary disease (COPD). However in these diseases especially IL-1β was found to be important. Nonetheless, we found in **chapter 6,** that IL-1α is the most important agonist on this pathway in allergic sensitization to HDM. We did not find a role for IL-1α in secondary responses to HDM, and also other groups have suggested a dual role for IL-1α [175]. IL-1α is suggested to be a danger signal released by necrotic cells, therefore inducing sterile inflammation [176]. We propose an autocrine loop of IL-1α leading to activation of lung epithelial cells and subsequent release of GM-CSF. Recruited dendritic cells or monocytes will gain their inflammatory state and induce Th_2 responses to HDM upon GM-CSF exposure. IL-1RI signaling on $\gamma\delta$ T cells and Th_{17} cells leads to release of IL-17 providing a tissue specific inflammation [177]. Blocking IL-1RI has already been proposed, in optimizing the blockade of this receptor [178, 179]. Since this pathway is necessary in viral clearance, therapeutic applications for treatment of asthma might rather lie downstream [180]. IL-1α-IL-1RI signaling leads to the release of GM-CSF and CCL20 as well as danger molecules such as IL-33. These cytokines influence the onset of asthma creating a pro-inflammatory environment. Chapter 6 shows that the sensitization to HDM in a model of allergic asthma is tightly controlled by airway epithelial cells, influencing the migration and activation of dendritic cells.

Allergic asthma is a disease of westernized countries. The 'hygiene hypothesis' states that viral or microbial infections in childhood decrease the incidence of allergic asthma [181]. Asthma,

atopy, eczema and hay-fever have a lower prevalence in overcrowding, unhygienic conditions and larger family size [182]. Specific immune activation pathways are not clear, however our data would suggest that instead of the inflammatory cells rather the AECs become irresponsive to allergens if encountered more infections in the past.

How are these data transferable to human lung disease ?

In our model of embolic antigen injection, we found that the size of inflammatory infiltrates decreases over time and is resolved after about 16 days (chapter 3). This model could therefore be a tool to study sarcoidosis since in patients the granulomas resolve over time in 80% of the cases, but in 10-30% results in fibrosis [183]. In cases when the immune response was initiated to the granuloma itself by antigen presenting cells (like macrophages or DCs) [184]. This would mean that recruited DCs are necessary for inducing an immune response that is predetermined to resolve, however if DCs were depleted no granulomas were formed in the first place. This suggests that many T cells inside a granuloma have an inherent anti-inflammatory character, something that has also been observed in patients with sarcoidosis and has been called the immune paradox of sarcoidosis [185]. Experimental and pre-clinical work has addressed the role of DCs in COPD and asthma [186]. However less is known about interstitial lung diseases like sarcoidosis and lung fibrosis. Sarcoidosis is generally accepted as a T cell mediated disorder [187]. In patients with active sarcoidosis increased numbers of regulatory T cells (Treg) are found suppressing early stages of granuloma formation [188, 189]. Tregs found within the granulomas showed less suppressing capacities beside their high IL-10 and TGF-β levels, compared to Tregs in the blood of the same patients [190]. In addition another study showed that TNFα levels are not completely impaired in sarcoidosis patients by their Tregs, while anti-TNFα has been shown to be very effective in this disease [185]. Preliminary murine experiments on the role of Tregs in granuloma formation, revealed depletion of Tregs at antigen exposure the infiltrate size was reduced. Treg depletion in model of RSV infection showed increased viral clearance [191]. In this RSV-model Tregs were found to express granzyme B. Granzyme B is found to be expressed by multinucleated giant cells, $CD4^+$ and $CD8^+$ T cells in sarcoidosis [192, 193]. Therefore granulomas could be rather maintained than suppressed by Tregs (expressing granzyme B). Subsequent analysis of levels of granzyme B on Tregs surrounding the beads in mice and experiments using granzyme B deficient Tregs could solve this question. Perhaps by targeting a specific DC subset in sarcoidosis would prevent Treg influx into the inflammatory area, leading to reduced risk in fibrosis development. It would be necessary to further study which DC subset is involved in antigen presentation and granuloma formation in sarcoidosis and fibrosis formation.

UDCA is used in clinics to treat patients with chronic cholestatic diseases like primary biliary cirrhosis (PBC). Patients with PBC show a considerable influx of eosinophils in the liver, while this is not seen in patients with viral chronic active hepatitis [194]. Screening of liver biopsies of PBC patients on cytokines showed an increase in IL-5, IL-6 and IFN-γ compared to controls or biopsies of patients with chronic hepatitis C [195]. Eosinophilia and increased Th_2 cytokines are also hallmarks of asthma (**chapter 1**). Especially Th_2 cytokine IL-13 and eosinophilia were reduced in our model after treatment with UDCA. An increase of exhaled nitric oxide (NO) has been shown to accompany eosinophilic inflammation. Exhaled NO increases during

exacerbation and decreases with recovery in patients with asthma. NO is also implicated in the Th_1/Th_2 balance, less NO leads to increased Th_1 expansion. UDCA could reduce NO production *in vitro* in human cell lines most likely at the level of nitric oxide synthase [196, 197]. We found that UDCA treatment lead more IFN-γ release by T cells, therefore a shift to Th_1. The exact mechanism of UDCA is not yet found in human or mouse studies, as well as DC function in liver diseases is not well established. Although many activated DCs ($CD11c^+$, $CD83^+$) are found in inflammatory regions in livers of PBC patients [198]. One could speculate that since DCs are found to be important for the structure of inflammatory regions [199], UDCA could act on the DCs in liver diseases as well, thereby treating the disease. If UDCA would be used as treatment in asthma, the next finding should be considered. Since 6 hours after stimulation of cells *in vitro*, UDCA lost its immune regulatory effects [197]. Therefore timing of UDCA treatment could be important. Only cells pretreated with UDCA or early in challenge an Th_2 reducing effect was found.

The epithelial network controlling asthma in humans has been proposed for a long time [200]. Air pollutants (ozone, NO, tobacco smoke) have been shown to worsen asthma. However reaction to air pollutants do not necessarily increase with the severity of asthma [201]. We have shown that TLR4 signaling on AECs is important in HDM-driven asthma [55]. CD14 is an adaptor molecule necessary for endotoxin/TLR4 signaling. TLR4 has been found to be associated with asthma [202]. Remarkably, CD14 polymorphism were found to be protective and also gene-environment correlations were found [203, 204]. Another study indicated that CD14 and TLR4 undergo endocytosis and is mainly involved in phagocytosis of microbes. Endocytosis is increased under exposure to inflammatory mediators and dependents on Syk signalling on DCs and macrophages [205]. Our results on UA support these findings. In a model using OVA alum, we showed that alum induced responses are dependent on UA. Alum injection *in vivo* increased endocytosis of OVA on peritoneal DCs [72]. It raises the question if a genetic defect in CD14 signaling (induced by polymorphisms) would prevent atopy or asthma? Therefore it could be very interesting to investigate CD14 expression on DCs after exposure to UA or HDM. On human lavage samples we found uric acid to be increased upon exposure to real life allergens. In addition blood samples of children with asthma and rhinitis show increased UA levels compared to controls [206]. Future research should be done on the role of UA in allergic asthmatics or rhinitis patients.

Downstream of TLR4 signaling we found besides the DC triggering by uric acid, activation of the IL-1RI pathway on AECs by IL-1α. Microarray data on human epithelial cell-line after exposure to grass pollen extract shows that IL-1α is one of the most up regulated genes after stimulation, together with pro-inflammatory IL-6, IL-8 and the natural occurring IL-1receptor antagonist (IL-1Ra) [207]. Polymorphisms within the IL-1Ra and IL-1α gene are associated with asthma and development of nasal polyps in asthmatics [208], which suggest that the role of these cytokines is transferable to patients. Downstream of IL-1RI signaling we found increased secretion of epithelial derived cytokines like IL-33 and GM-CSF. Recent genetic studies on asthma have identified single nucleotide polymorphisms (SNPs) in the gene coding for IL-33 receptor IL-1RL1 (ST2 receptor) as well as in the IL-33 locus itself [209]. Polymorphism in the CSF2 gene, which is the gene encoding for GM-CSF were found to be correlating with asthma

in Swiss and atopic dermatitis in Canadian children [210, 211]. Regulatory gene region of MCP-1 polymorphism were found as well [212] however not much is known so far on CCL20. Our data show a timeline of the cytokine cascade which follows upon HDM exposure.

Future perspectives

DCs are very important in antigen presentation, which is known for inhaled allergens. Here we show for the first time, DCs presenting bloodborn antigens in the MLN. Targeting DCs or depleting them, leads to reduced inflammatory lesions and in asthma to reduced eosinophilia and Th_2 responses. We have unraveled an important role of airway epithelial cells in controlling DC activation by release of pro-inflammatory cytokines and chemokines downstream of TLR4/IL-1RI axis. In the future we will analyze how environmental triggers like infections, air pollutants (diesel, ozone etc.) affect the balance of these cytokines and thereby the function of DCs. This raises the possibility that the epithelium is genetically modified by these environmental triggers. A high dose of TLR4 ligand LPS leads to tolerance of the epithelium for weeks [213]. It would therefore be very interesting which intracellular inhibitors are induced by LPS and how long tolerance sustains. Preliminary data showed that inhibitory TNFα induced protein 3 (TNFAIP3 or A20) is increased in the lung after exposure to CPG (TLR9 agonist) and LPS.

A20 is a negatively regulator of TLR/IL-1RI and TNF-receptor signaling. Recently multiple SNPs in the TNFAIP3 interacting protein 1 (TNIP1) gene, which interact with A20 were associated with asthma [214]. A20 therefore is a possible candidate for therapeutic implications on structural cells. To investigate A20 regulation in patients, analysis should be done on peripheral tissue, like biopsies instead of blood or lavage to be able to analyze gene expression and modifications upon allergen exposure and after treatment.

Concluding remark

DCs and AECs interact with each other to induce an immune response to foreign particles. Research nowadays focusses on the origin and function of DC subsets and the PRR signaling pathways involved. Basal research will help finding a new therapeutic strategy which involves either instructing AECs or directly targeting DCs. For allergic asthma there is no direct cure yet, however understanding the mechanism of sensitization will lead to better medication in the future. Since the often prescribed corticosteroids are after all only fighting the symptoms and not the onset of the disease.

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Abbreviations

AEC	airway epithelial cell
AGR2	anterior gradient homolog 2
AHR	airway hyperresponsiveness
AIM2	absent in melanoma 2
APC	antigen presenting cell
ASC	apoptosis-associated speck-like protein containing a caspase recruitment
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BHR	broncho-hyperresponsiveness
BMDCs	bone marrow-derived dendritic cells
CCL	chemokine (C-C motif) ligand
cDC	classical dendritic cell
CDCA	chenodeoxycholic acid
CDP	common DC precursor
COPD	chronic obstructive pulmonary disease
CPG-ODN	CpG oligodeoxynucleotides
d	day(s)
DAMP	danger associated molecular patterns
DBEC	diseased bronchial epithelial cell
DC	dendritic cell
	domain
DT	diphtheria toxin
DTR	diphtheria toxin receptor
EC	epithelial cell
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmatic reticulum
FACS	fluorescent activated cell sorting
GCM	goblet cell metaplasia
GI	gastrointestinal
GM-CSF	granulocyte macrophage-colony stimulating factor
GMP	granulocyte-myeloid precursor
GR	glucocorticoid receptor
GWAS	genome wide association study
HDM	house dust mite
HMGB1	high mobility group box 1 protein
HSC	hematopoietic stem cell
HSP	heat shock proteins
i.n.	intranasally
i.p.	intraperitoneally
i.t.	intratracheally
i.v.	intravenously
ICS	inhaled corticosteroids
IgE	immunoglobulin E
IL	interleukin
IL-1Ra	IL-1 receptor antagonist
IL-1RI	IL-1 receptor 1
IRF3	interferon regulatory transcription factor 3
ITIM	immune receptor tyrosine-based inhibitory motif

LP	lymphoid precursor
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LSPs	leaderless secretory proteins
MCH	methacholine
MCP-1	monocyte chemotactic protein-1
MD2	myeloid differentiation protein 2
MHC	major histocompatibility complex
MLN	mediastinal lymph node
mo-DC	monocyte-derived dendritic cell
MUC5A	mucin-5A
MYD88	myeloid differentiation primary response 88
NALP3	NACHT, LRR and PYD domains-containing protein 3
NBEC	normal bronchial epithelial cell
NF-κB	nuclear factor- kappa B
NLR	NOD like receptors
NO	nitric oxide
OB	OVA coated Sepharose bead
OVA	ovalbumin
PAMP	pathogen associated molecular patterns
PAR	protease-activated receptor
PAS	periodic acid Schiff
PBC	primary biliary cirrhosis
PBMCs	peripheral blood mononuclear cells
PD-1	programmed death -1
pDC	plasmacytoid dendritic cell
PMA	phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic-polycytidylic acid
PPARs	Peroxisome proliferator-activated receptors
PPARγ	proliferator-activated receptor-γ
pre-cDC	preclassical dendritic cell
PRR	pattern recognition receptor
PYHIN	pyrin and HIN domain-containing protein
RLR	RIG like receptors
ROS	reactive oxygen species
SHP	small heterodimer partner
SNP	single nucleotide polymorphisms
Tg	transgenic
Th	T helper cell
TLR	toll like receptor
TNFα	tumor necrosis factor- alpha
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
TSLPR	TSLP receptor
TUDCA	tauroursodeoxycholic acid
UA	uric acid
UB	uncoated Sepharose bead
UDCA	ursodeoxycholic acid
UPR	unfolded-protein response
XOR	xanthine oxido reductase

Curriculum Vitae

The author is born on August 17, 1980 in Dongen (the Netherlands). In 1998, she graduated High school (VWO) at the Cambeurcollege in Dongen. In 1999 she started with Biology and Laboratory-school at Hogeschool Brabant in Etten-Leur. Her internship on mast cell activation of allergic rhinitis patients was performed at the ENT-department of the Medical Faculty at the ErasmusMC in Rotterdam (The Netherlands) under supervision of Dr. Alex KleinJan. In October 2002 she obtained Bachelor-degree. She started to work as a research technician in December 2002 in the laboratory of Pulmonary Medicine at the ErasmusMC Medical Faculty. Under supervision of Prof. Dr. Bart Lambrecht and Dr. Alex KleinJan, she worked on several projects studying the role of dendritic cells in allergic asthma, allergic rhinitis and transplantation and guided 5 trainees during their internship. In October 2007 she started at Ghent University as a PhD-student. Since than she helped starting a new research group within the department of Pulmonary Medicine of Ghent University together with (co-)promotors Hamida Hammad and Bart Lambrecht. In January 2012 this research group moved to the Department for Molecular Biomedical Research (DMBR) at VIB (Zwijnaarde). In January 2008 she started her PhD-research, leading to this thesis.

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